Manuscript Details

Manuscript number JEP_2018_1967_R1

Title In vitro studies on anti-inflammatory activities of kiwifruit peel extract in human THP-1 monocytes

Article type Research Paper

Abstract

Ethnopharmacological relevance: Kiwifruit is native to eastern China and many are the references about the consumption of fruits and fruits extracts of the Actinidia plants in Chinese traditional medicine as therapeutic food supplements to prevent and/or counteract numerous disorders including inflammation-related diseases like cancer. Aim of the study: Aim of the present work was to obtain a kiwifruit peel extract, rich in polyphenols, and to explore the anti-inflammatory potential by analyzing its capability to target multiple pathways involved in monocyte-mediated inflammatory response. Materials and Methods: The extract was obtained from the fruit peel of Actinidia deliciosa (A.Chev.) C.F.Liang & A.R.Ferguson, cv Hayward and characterized by HPLC-DAD-ESI-MS. Lipopolysaccharide-stimulated THP-1 monocytes were used as a model of human inflammation in vitro. Results: Analytical data evidenced that procyanidins resulted the main polyphenols present in the extract, representing the 92% w/w of the total. The extract inhibited the production of inflammatory molecules such as IL-6, IL-1β, TNF-□ pro-inflammatory cytokines, HMGB1 danger signal and granzyme B serine protease by activated monocytes. In particular, an inhibitory activity of 81%, 68%, 63%, 76% and 60% on the extracellular release of IL-6, IL-1□, TNF-□, HMGB1 and granzyme B, respectively, was observed by western blot analysis. Moreover, the extract prevented STAT3 activation and promoted autophagy. Conclusions: The reported findings demonstrated a strong and broad anti-inflammatory profile of the kiwifruit peel extract, which makes it a promising preventive and therapeutic natural ingredient for nutraceutical, cosmetic and pharmaceutical formulations to counteract multiple inflammatory disorders.

Keywords kiwifruit (Actinidia deliciosa, cv Hayward) peel extract, procyanidins, inflammation, STAT3, autophagy

Taxonomy Biopharmaceuticals, Pharmaceutical Compounds Formulation

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24th December 2018

Dear Prof. Fernandes,

Please, find below our answers to the comments we received from the Reviewers concerning our manuscript entitled *"In vitro studies on anti-inflammatory activities of kiwifruit peel extract in human THP-1 monocytes"* by D. D'Eliseo, E. Pannucci, R. Bernini, M. Campo, A. Romani, L. Santi and F. Velotti, submitted for publication in *Journal of Ethnopharmacology* (Ref: **JEP_2018_1967**). Any modification to the manuscript is highlighted in green in the text and specifically in the relative answer.

We sincerely thank you for your time and consideration,

Prof. Roberta Bernini

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REVIEWER 1

The manuscript by Donatella D'Eliseo et al. focused on the study of anti-inflammatory activities of kiwifruit peel extract in human THP-1 monocytes. Main problems:

1. Please replace the protein blots in Figure 1 with a clearer band.

As requested, we have replaced the protein blots in Figure 2 (referred to protein blots) with clearer bands (mostly for panel b). In addition, the inhibitory effect by the extract on the release of pro-inflammatory cytokines in the conditioned media (Figure 2, panel b) was confirmed by the MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead panel analysis (Figure 2, panel c). (See *Figure 2*).

REVIEWER 2

In the Ms "In vitro studies on anti-inflammatory activities of kiwifruit peel extract in human THP-1 monocytes"

Abstract:

1. What do the phrase "inflammation-related diseases like cancer" means?

Cancer is a disease related to inflammation, in that many cancers arise from sites of infection, chronic irritation and inflammation. It is now becoming clear that the tumor microenvironment, which is largely orchestrated by inflammatory cells, is an indispensable participant in the neoplastic process, fostering proliferation, survival and migration. In addition, tumor cells have co-opted some of the signaling molecules of the innate immune system, such as selectins, chemokines and their receptors for invasion, migration and metastasis (*Coussens L.M., Werb, Z. 2002. Inflammation and cancer. Nature, 420, 860-867; Hanahan, D., Weinberg, R.A. 2011. Hallmarks of cancer: the next generation. Cell. 144, 646-674; Suarez-Carmona, M., Lesage, J. Cataldo, D., Gilles, C. 2017. EMT and inflammation: inseparable actors of cancer progression. Mol. Oncol. 11, 805-823*).

2. How THP-1 monocytes tested in vitro could be related to in vivo effects?

THP-1 cell line has unique characteristics as a model to investigate/estimate immune-modulating effects of compounds in both activated and resting conditions of the cells, and the THP-1 response can hint to potential responses that might occur *ex vivo* or *in vivo* (*Chanput, W., Mes, J.J., Wichers, H.J. 2014. THP-1 cell line: an in vitro cell model for immune modulation approach. Int. Immunopharmacol. 23, 37-45; Qin, Z. 2012. The use of THP-1 cells as a model for mimicking the function and regulation of monocytes and macrophages in the vasculature. Atherosclerosis 221, 2-11*). However, the conclusion of studies employing THP-1 cells requires further verification using *in vivo* models. Therefore, we added the following sentence:
"However, this assumption requires further verification using *in vivo* models." (See *4. Conclusions*).

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3. Add data in results and not only "the extract inhibited production of…". Add numbers

As requested, we added the percentages of inhibition as follows:

"In particular, an inhibitory activity of 81%, 68%, 63%, 76% and 60% on the extracellular release of IL-6, IL-1 β, TNF-α, HMGB1 and granzyme B, respectively, was observed by western blot analysis." (See *Abstract, Results*).

Introduction:

4. JEP is a journal directed to the comprobation of the traditional use of plants. In this regards, authors must clearly indicate which part of the fruit is used in traditional medicine. "kiwifruit has known an ever-wider use in traditional medicine for its ability…". Which part? Which part of the plant is used in TM?

Actinidia plant has been clearly used in traditional medicine; fruits and fruits extracts were consumed as therapeutic food supplements for the treatment of several diseases (*Motohashi, N., Shirataki, Y.; Kawase, M.; Tani, S.; Sakagami, H., Satoh, K., Kurihara, T., Nakashima, H., Muacis, I., Varga A., Molnar J. 2002. Cancer prevention and therapy with kiwifruit in Chinese folklore medicine: a study of kiwifruit extracts. J. Ethnopharmacol. 81, 357-364; Singletary, K. 2012. Kiwifruit. Overview of potential health benefits. Nutr. Today, 47, 133-147; Sun, S., Xu, H., Ngeh, L. 2012. The evaluation of Chinese therapeutic food per the treatment of moderate disylipidemia. Evidence-Based Complementary and Alternative Medicine. 1-11*). Although not always directly specified, it is indeed likely that the mesocarp and endocarp of the berry was consumed while the peel consisting of the esocarp of the fruit was discarded. However, since the peel is so intimately connected to the edible parts used in TM and also because it has been scarcely investigated, never for the biological activities we studied, we considered our research of interest and in line with the aim and scope of the Journal. This reasoning seems also in agreement with Motohashi and coauthors, which published an interesting paper using a novel variety of kiwifruit, a recently introduced gold cultivar, indeed never used directly in TM (*Motohashi, N., Shirataki, Y.; Kawase, M.; Tani, S.; Sakagami, H., Satoh, K., Kurihara, T., Nakashima, H., Muacis, I., Varga A., Molnar J. 2002. Cancer prevention and therapy with kiwifruit in Chinese folklore medicine: a study of kiwifruit extracts. J. Ethnopharmacol. 81, 357-364*).

Finally, this issue was already raised by the Editor during our submission last June, and after we presented our motivations (see presentation letter to the Editor reported at the bottom) the manuscript was granted to proceed with the peer review procedure.

Nevertheless, we totally agree that any ambiguity concerning the part of the fruit that was used must be corrected, and accordingly, we introduced some changes in Abstract, Introduction and Conclusions sections.

We also removed some references about the use of *Citrus unshiu*, banana (*Musa* spp), hawthorn (*Crataegus pinnatifida*), grape (*Vitis vinifera)*, orange (*Citrus sinensis* L.), and pomegranate (*Punica granatum*) peel in traditional medicine (Park et al., 2018; Pereira et al., 2015; Wu et al. 2017; Shirataki et al. 2000; Erukainure et al. 2016); Khan et al. 2017).

In addition, we have also added the following references:

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- Yang, J.-X. 1981. Chinese pharmaceuticals for cancers. General people's health publishers. Peking, 121-122.
- Zhi, C.-J. 1980. Chinese anti-cancer agents. Hua-lian Publishers, Taipei, p.1, 74-75.

Based on these changes, the final versions concerning this aspect are the following:

- Kiwifruit is native to eastern China and many are the references about the consumption of fruits and fruits extracts of the *Actinidia* plants in Chinese traditional medicine as therapeutic food supplements to prevent and/or counteract numerous disorders including inflammation-related diseases like cancer. (see *Abstract, Ethnopharmacological relevance*).
- The use of kiwifruit in Chinese traditional medicine is well documented since the 1400's A.D. while the first recorded reference on the effects against a "cancer-like" disease dates even back to the 720's B.C. (Motohashi et al., 2002; Singletary, K., 2012; Sun et al., 2012). Over the years, kiwifruit has known an ever-wider use in traditional medicine to prevent cardiovascular and degenerative diseases, to relieve disorders caused from dyspepsia, rheumatism, digestive problems and to treat stomach, liver and rectal cancer (Zhi, 1980; Yang, 1981). (See *1. Introduction).*
- Based on the consideration that the peel is intimately connected to the pulp, which is the edible part of kiwifruits largely used in Chinese traditional medicine, and the lack of the literature on the biological activities of kiwifruits peel, the aim of this work was to investigate the *in vitro* anti-inflammatory activity of a peel extract rich in polyphenols obtained from *Actinidia deliciosa* on activated human THP-1 monocytes, through the analysis of its capability to target multiple processes involved in monocyte-mediated inflammation. (See *1. Introduction).*
- In summary, this study has proved for the first time that an extract rich in procyanidins, obtained from the peel of kiwifruits, is a suppressor of the production of a number of inflammatory mediators such as pro-inflammatory cytokines, HMGB1, granzyme B and STAT3 and a promoter of autophagy on activated human THP-1 monocytes. (See *4. Conclusions).*

5. Cancer is not an inflammatory disease.

We have already answered above, in the answer to question 1. Here, we can specify the molecular and cellular circuits linking inflammation and cancer. Two pathways have been schematically identified: (i) the intrinsic pathway, where genetic events causing cancer initiate the expression of inflammation-related programs that guide the construction of an inflammatory microenvironment; for example, oncogenes share the capacity to orchestrate pro-inflammatory circuits; (ii) the extrinsic pathway, where chronic inflammatory conditions facilitate cancer development and progression, including infections (e.g. *Helicobacter pylori* for gastric cancer and mucosal lymphoma; papilloma virus and hepatitis viruses for cervical and liver carcinoma, respectively), autoimmune diseases (e.g. inflammatory bowel disease for colon cancer) and inflammatory conditions of uncertain origin (e.g. prostatitis for prostate cancer) (*Hanahan, D., Weinberg, R.A. 2011. Hallmarks of cancer: the next generation. Cell. 144, 646-674).*

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6. Monocytes are not the essential players in inflammation. There are several cell types. i.e. neutrophils, lymphocytes….

Inflammation is a progressive process, which is initiated in response to tissue injury or infection. Thus, acute inflammation is associated with inflammatory monocytes/macrophages and high levels of polymorphonuclear cells, particularly neutrophils, whereas chronic or adaptive immune inflammation has higher levels of mononuclear cells, macrophages, T- and B-lymphocytes. Since monocyte-derived macrophages orchestrate both the initiation and the resolution of inflammation, they contribute to the pathogenesis and progression of multiple diseases (*Karlmark, K.R., Tacke, F., Dunay, I.R. 2012. Monocytes in health and disease. Eur. J. Microbiol. Immunol. (Bp). 2, 97-102; Jou, I.M., Lin,C.F., Tsai, K.J., Wei, S.J. 2013. Macrophage-mediated inflammatory disorders. Mediators Inflamm. 316-482*), including liver fibrosis (*Tacke F. 2017. Targeting hepatic macrophages to treat liver diseases. J. Hepatol. 66, 1300-1312*), atherosclerosis (*Woollard, K.J., Geissmann, F. Monocytes in atherosclerosis: subsets and functions. Nature Rev. Cardiol. 7, 77-86*), and tumor development and progression *(Kitamura, T., Qian, B.Z., Pollard, J.W. 2015. Immune cell promotion of metastasis. Nat. Rev. Immunol. 15, 73-86).* Therefore, monocytes/macrophages represent an interesting target for designing anti-inflammatory therapeutic strategies.

7. "Several studies reported…": and only one reference was added!

As requested by the Reviewer, we have added some references cited in other parts of the manuscript as follows:

"Several studies reported that polyphenols (Andujar et al., 2011; Pietrocola et al., 2012; Bernini et al., 2013 and 2015; Rosillo et al., 2014; Liu et al., 2018) …….." (See *1.Introduction*).

8. There is only one ref for procyanidins anti-inflammatory effect?

The reference included in the manuscript is a review article. However, as requested by the Reviewer, we have added the selected references:

- Bak, M.-J.; Truong, V.L.; Kang, H.S.; Jun, M. and Jeong, W.S. 2013. Anti-inflammatory effect of procyanidins from wild grape (*Vitis amurensis*) seeds in LPS-induced RAW 264.7 cells. Oxidative Medicine and Cellular Longevity. http://dx.doi.org/10.1155/2013/409321.
- De la Iglesia, R.; Milagro, F. I.; Campion, J.; Boque, N.; Martinez, J. A. 2010. Healthy properties of proanthocyanidins. Biofactors, 36, 159-168

and we have modified the text as follows:

"and, in particular, procyanidins exerted an anti-inflammatory activity (Bak et al., 2013; De La Iglesia et al., 2010; Martinez-Micaelo et al., 2012)". (See *1.Introduction*).

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9. The fact that other peel extracts are used in TM do not justify the use and/or test of kiwi.
We agree with the Reviewer and we then added some changes in Abstract, Introduction, Conclusions and References sections (see answer to question 4).

Methods:
10. Necessary voucher number, herbarium where it was deposited and name of botanic!
The kiwifruit variety is the Hayward the most cultivated green fruit cultivar in the world and Italy. We obtained the fruits from a professional orchard, which for productive reasons is not only made of plant trees belonging to the same specie and cultivar but that are possibly all clones of the same parent tree. The plant botanical name is (*Actinidia deliciosa* (A.Chev.) C.F.Liang & A.R.Ferguson, cv Hayward) this information is included in the manuscript. We have also specified that *Actinidia deliciosa* fruits were collected from Stefano Della Bianca orchard located at Cisterna di Latina (Latina, Italy), giving the precise GPS coordinates (Latitude: 41°31'42.8"N; Longitude: 12°47'31.3"E). A representative of the cultivar has been transplanted in the "Orto dei semplici" section of the "Angelo Rambelli" Botanical Garden of the University of Tuscia, under the accession GS159. (See *2.1 Plant Material*).

11. Add number of cell line deposited in ATCC
As requested, we have added the ATCC designation for THP-1 cells as follows:
"ATCC® TIB-202™" (See *2.3 Cell culture*).

12. Add type of LPS used
We have added the type of LPS used as follows:
"LPS from Escherichia coli O55:B5 (L2880 Sigma)" (See *2.4 Cell treatments*).

13. Clarify what is "ethanolic solution (Control)".
"Ethanolic solution (Control)" is our vehicle alone, corresponding to ethanolic solution containing the extract for cell treatment. Therefore, we have modified the text as follows:
"THP-1 cells were incubated with ethanolic solution alone (Control) or with the extract, solubilized in ethanolic solution, at the indicated doses, for the indicated times". (See *2.4 Cell treatments*)

14. 1g of powder was extracted with ethanol/water (70/30). The supernatant was collected. How authors transformed this volume (μL) in μg?
The extract was prepared by maintaining under stirring an exact weight of vegetal matrix (1.0 g) in an exact volume of solvent (50.0 mL of $EtOH/H_2O$=70/30 v/v). The final extract was characterized by HPLC/DAD/MS. The analytical data are expressed by the instrument as mg/mL of individual compounds in the extract. In Table 1, they are expressed as mg per gram of peel considering that each mL of extract is referred to 0.02 g of kiwifruit peel (1.0 g/50.0 mL).
Consequently, we have modified the text as follows:
"Results are given as means and expressed as mg per gram of peel (Table 1) considering that, each mL of extract is referred to 0.02 g of kiwifruit peel; standard error was always <5% (Pinelli et al., 2013)". (See *2.2 Kiwifruit peel extract preparation and characterization*).

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15. To incubate with cells?

We agree with the Reviewer that an explanation about this point is necessary. Thus, we added the following text:
"For the biological activities, a stock solution of the extract solubilized in ethanol/water=70/30, corresponding to a concentration of 12 mg/mL, was expressly prepared as briefly described. From a sample of extract solution, the solvent was removed under reduced pressure by a rotary evaporator (Heidolph, Germany) to afford a powder that was weighted and solubilized with a known volume of ethanol/water=70/30 to obtain 12 mg/mL (stock solution). Then, cells were incubated with the indicated concentrations of the extract (25, 50, 100 and 200 µg/mL), by diluting the stock solution in the cell culture medium. The final concentration of ethanol in the cell culture medium, for both test and corresponding control experiment, was always <1.6 %, compatible with cell viability (assessed by the trypan blue dye exclusion assay) and function (assessed by the analysis of cell proliferation)". (See *2.2 Kiwifruit peel extract preparation and characterization*).

16. How authors calculate the concentrations used?

As reported above (answer to question 15), the concentrations used were calculated by diluting the stock solution (12 mg/mL of the extract) with the cell culture medium.

17. How authors prepared the control group in fig 1?

The control group in Figure 1 is the ethanolic solution alone, corresponding to the ethanolic solution containing the highest dose of the extract (200 µg/mL).

18. It seems that cell numbers increased with 25 and 50 ug/ml after 24h. how explains it?

It might seem that cell number increased, because the standard deviation (S.D.) of the three independent experiments performed with 25 µg/ml is higher than that with 12.5 µg/ml or control. However, the number of cells cultured in the presence of 25 and 50 ug/ml for 24h was not statistically different from that of the control.

19. How explains the fact that with 100 and 200 we observe reduction in cell number and not in cell viability?

The cell number is very likely reduced due to the inhibition of cell proliferation and not to the induction of cell death. We have already written as follows: "Moreover, the evaluation of cell growth showed that the cell number was not affected up to an extract concentration of 100 µg/mL and then decreased in a dose dependent manner (Fig. 1B), suggesting that a proliferative arrest might occur at high concentrations." (See *3.2. Effects of kiwifruit peel extract on THP-1 cell viability and proliferation*).

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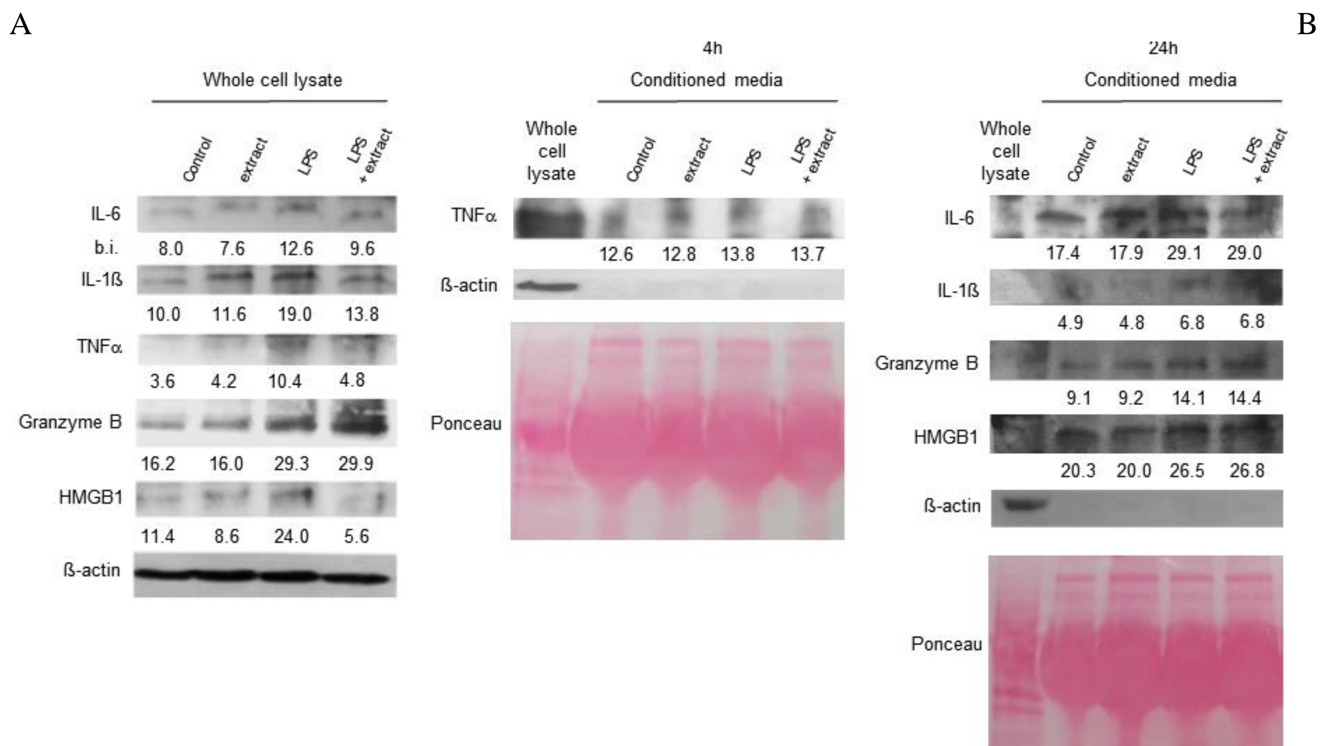
20. In fig 2 authors showed data with one concentration (50 ug). As it demonstrated significant effect it is necessary to show lower concentrations. The fact that 50 ug had effect it is important to demonstrate that lower conc. do not present (or present) effect

We agree with the Reviewer and we have performed experiments treating THP-1 cells with a lower concentration, corresponding to 25 µg/mL, for 24 h. A representative experiment is shown below:



An inhibitory effect, lower than that induced by the extract at the higher dose of 50 µg/mL (see manuscript, Figure 2 panel a), was observed on the intracellular content of inflammatory molecules (except of granzyme B) in LPS-stimulated THP-1 cells (panel A). However, no inhibitory effect was observed on the extracellular content of inflammatory molecules, either by western blot (panel B) or by Luminex multiple assay (data not shown) analyses. Therefore, at a lower extract concentration, an effect is present and is dose-dependent in terms of inhibition of the intracellular production of inflammatory molecules by LPS-stimulated cells (panel A), but not in terms of inhibition of extracellular release of these molecules (panels B). To note, in contrast to Figure 2 panels a-b in the manuscript, no inhibitory effect was observed on both the intracellular and extracellular content of inflammatory molecules in unstimulated THP-1 cells (panels A and B). This result might be explained by the fact that, since the production of inflammatory molecules by unstimulated THP-1 cells is clearly lower than that by cells undergoing LPS stimulation, an inhibitory effect (likely induced at a lower extent by a lower dose of extract) on a low amount of molecules is not detectable. This assumption might also explain why we observed an inhibitory

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effect on the intracellular but not on the extracellular content of inflammatory molecules by a lower dose of extract in LPS-stimulated THP-1 cells: the amount of extracellular released inflammatory molecules is lower than that of intracellular molecules; thus, the inhibitory effect (induced at a lower extent by a lower dose of extract) on a low amount of extracellular molecules might not be detectable. To overcome this case, we tried to improve protein detection sensitivity in the extracellular milieu, by performing western blot using prolonged incubations with antibodies, longer exposure times of the film or using Amicon® Ultra Centrifugal filters for protein concentration in the conditioned media, without success.
In conclusion, since we could not establish whether the absence of the inhibitory effect by the lower dose of extract on the extracellular release of pro-inflammatory molecules by LPS-stimulated cells was due to the absence of this effect or more likely to a low amount of extracellular molecules (for which an inhibitory effect was not detectable), we consider that is not appropriate to add and/or discuss these experiments in the manuscript. In any case, although a dose-dependent effect of the extract would have been interesting to establish, the aim of the present study was to find an experimental condition, in an *in vitro* inflammatory model, to bring out the anti-inflammatory activity of a kiwifruit peel extract.

21. "… as well as anti-cancer natural…": the conclusion that the extract can present anti-cancer effect is too speculative. Any data was presented.
We showed that the extract inhibited the constitutive STAT-3 level in unstimulated THP-1 cells, that are monocytic leukemia cells (AML). For this reason, in the text (see *3.6. Kiwifruit peel extract prevented STAT3 activation*) we have already written: "In addition, an important role of STAT3 has been identified in the development of different cancers, including AML (Redell et al., 2011). Therefore, our result, showing the inhibition of the constitutive level of STAT3 in unstimulated THP-1 monocytic leukemia cells, suggests that the extract might also exert anti-cancer activity in AML. Considering all these findings, we think that the extract might represent a potential promising candidate to target the IL-6/STAT3 pathway in inflammatory diseases and cancer."
However, since our conclusion seems too speculative, we have deleted ".. as well as anti-cancer.." (see *5. Conclusions*).

In summary: the Ms is interesting. However, authors must include data with lower concentrations to demonstrate the dose response effect (or it absence). At least 3 doses (or concentrations) must be presented. It is also too speculative to indicate that the extract could be used in cancer. It is also too speculative to conclude that IN VITRO data suggests that the extract can present IN VIVO effect!
We appreciated that the Reviewer has considered our manuscript interesting and, according to the final comments, we have:
(i) performed, shown and discussed experiments with a lower concentration (see question 20);
(ii) deleted the sentence concerning our speculation on the potential anti-cancer activity of the extract (see question 21).
(iii) added in the Conclusions section the following sentence: "However, this assumption requires further verification using in vivo models."

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23th June 2018

Dear Editor,

Thank you for the possibility that you offer us to resubmit our manuscript entitled *"In vitro* studies on anti-inflammatory activities of kiwifruit peel extract in human THP-1 monocytes*"* by D. D'Eliseo, E. Pannucci, R. Bernini, M. Campo, A. Romani, L. Santi and F. Velotti for publication in *Journal of Ethnopharmacology* (**Ref: JEP_2018_1967**).

The starting points of our study, described in the Introduction of the revised manuscript, are the following literature data:

(a) the use of kiwifruit in Chinese traditional medicine

Motohashi, N., Shirataki, Y.; Kawase, M.; Tani, S.; Sakagami, H., Satoh, K., Kurihara, T., Nakashima, H., Muacis, I., Varga A., Molnar J. Cancer prevention and therapy with kiwifruit in Chinese folklore medicine: a study of kiwifruit extracts. J. Ethnopharmacol. 2002, 81, 357-364 and references therein.

Sun, S., Xu, H., Ngeh, L. 2012. The evaluation of Chinese therapeutic food per the treatment of moderate disylipidemia. Evidence-Based Complementary and Alternative Medicine. 1-11 and references therein.

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Based on these assumptions, in our manuscript we reported a study on the anti-inflammatory activity exerted by a polyphenolic extract obtained from kiwifruit peel by analyzing its capability to target multiple critical biological processes involved in monocyte-mediated inflammatory response. In particular, the extract was obtained from green fruits (*Actinidia deliciosa*, cv Hayward) peel and characterized by HPLC-DAD-ESI-MS. The analytical data evidenced that the extract was rich in procyanidins. Lipopolysaccharide-stimulated THP-1 monocytes were used as a model of human inflammation *in vitro*. The experimental data showed that the extract inhibited the production of multiple inflammatory mediators from activated monocytes, such as IL-6, IL-1β, TNF-α pro-inflammatory cytokines, HMGB1 danger signal and granzyme B serine protease. Moreover, it prevented STAT3 activation and promoted autophagy.

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In conclusion, we consider that our findings are relevant, since they indicate that, **similarly to what is already known for pulp and peel extracts of several fruits used in traditional medicine, kiwifruits peel extract has the potential to be envisioned as a therapeutic agent to prevent and/or counteract inflammation-associated diseases. Moreover, it is noteworthy that the main use of kiwifruit in Chinese traditional medicine is related to cancer prevention and treatment and nowadays the connection between chronic inflammation and cancer development is undoubtedly established.**

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Best regards,
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In vitro studies on anti-inflammatory activities of kiwifruit peel extract in human THP-1 monocytes

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Abstract
Ethnopharmacological relevance: Kiwifruit is native to eastern China and many are the references ~~of the use of this edible fruit and its extracts~~ about the consumption of fruits and fruits extracts of the *Actinidia* plants in Chinese traditional medicine as therapeutic food supplements to prevent and/or counteract numerous disorders including inflammation-related diseases like cancer. ~~Nevertheless, polyphenol-rich peel extracts obtained from several fruits are therapeutic food supplements known to the traditional medicine.~~
Aim of the study: Aim of the present work was to obtain a kiwifruit peel extract, rich in polyphenols, and to explore the anti-inflammatory potential by analyzing its capability to target multiple pathways involved in monocyte-mediated inflammatory response.
Materials and Methods: The extract was obtained from the fruit peel of *Actinidia deliciosa* (A.Chev.) C.F.Liang & A.R.Ferguson*,* cv Hayward and characterized by HPLC-DAD-ESI-MS. Lipopolysaccharide-stimulated THP-1 monocytes were used as a model of human inflammation *in vitro*.
Results: Analytical data evidenced that procyanidins resulted the main polyphenols present in the extract, representing the 92% w/w of the total. The extract inhibited the production of inflammatory molecules such as IL-6, IL-1β, TNF-α pro-inflammatory cytokines, HMGB1 danger signal and granzyme B serine protease by activated monocytes. In particular, an inhibitory activity of 81%, 68%, 63%, 76% and 60% on the extracellular release of IL-6, IL-1β, TNF-α, HMGB1 and granzyme B, respectively, was observed by western blot analysis. Moreover, the extract prevented STAT3 activation and promoted autophagy.
Conclusions: The reported findings demonstrated a strong and broad anti-inflammatory profile of the kiwifruit peel extract, which makes it a promising preventive and therapeutic natural ingredient for nutraceutical, cosmetic and pharmaceutical formulations to counteract multiple inflammatory disorders.

Keywords: kiwifruit (*Actinidia deliciosa,* cv Hayward) peel extract, procyanidins, inflammation, STAT3, autophagy.

1. Introduction

~~Natural products from medicinal plants are therapeutic agents widely used in traditional medicine for the treatment of several inflammation-related diseases including cancer (Cragg et al., 2009). In additiom, fruit extracts including both pulp and peel extracts from *Citrus unshiu* Markowich (Park et al., 2018), banana (*Musa* spp) (Pereira et al., 2015), hawthorn (*Crataegus pinnatifida*) (Wu et al. 2017), grape (*Vitis vinifera)* (Shirataki et al. 2000), orange (*Citrus sinensis* L.) (Erukainure et al. 2016), pomegranate (*Punica granatum*) (Khan et al. 2017) and kiwifruits (*Actinidia deliciosa*), are precious sources of polyphenols, and are used in traditional medicine as therapeutic food supplements.~~

Kiwifruit is the edible berry of the *Actinidia* plant (Actinidiaceae). The most commonly consumed is the green berry of the *Actinidia deliciosa* (A.Chev.) C.F.Liang & A.R.Ferguson*,* cv Hayward. According to data provided by the Food and Agriculture Organization (FAO) of the United States, kiwifruits cultivation is relevant in China (the native country), Italy, New Zealand, Chile and Greece, which represent the five largest producers in the world.

The use of kiwifruit in Chinese traditional medicine is well documented since the 1400’s A.D. while the first recorded reference on the effects against a “cancer-like” disease dates even back to the 720’s B.C. (Motohashi et al., 2002; Singletary, K., 2012; Sun et al., 2012). Over the years, kiwifruit has known an ever-wider use in traditional medicine to prevent cardiovascular and degenerative diseases, to relieve disorders caused from dyspepsia, rheumatism, digestive problems and to treat stomach, liver and rectal cancer (Zhi, 1980; Yang, 1981). ~~for its ability to prevent and/or counteract different disorders.~~

More recently, several studies have been focused on the biological activities of the kiwifruit, by individually analyzing extracts derived from either the fruit (Motohashi et al., 2002; Hunter et al., 2011) or the peel (Motohashi et al., 2001; Yang et al., 2013; Lee et al., 2014). However, to the best of our knowledge, the anti-inflammatory effects of kiwifruit peel extract have never been investigated so far.

Inflammation is the body's normal response to injuries or infections, but, when excessive or persistent, it causes and advances many serious inflammatory diseases including cancer (Lansky et al., 2007; Cragg et al., 2009; Martinez-Micaelo et al., 2012; Bernini et al., 2013 and 2015). Monocytes and monocyte-derived cells represent critical players in the orchestration of the inflammatory response, representing thus key therapeutic targets for disease treatment. Several studies reported that polyphenols (Andujar et al., 2011; Pietrocola et al., 2012; Bernini et al., 2013 and 2015; Rosillo et al., 2014; Liu et al., 2018), and, in particular, procyanidins exerted an anti-inflammatory activity (Bak et al., 2013; De La Iglesia et al., 2010; Martinez-Micaelo et al., 2012).

~~Based on the clear use in folk medicine of kiwifruits and, in particular, of peel extracts of several fruits,~~ Based on the consideration that the peel is intimately connected to the pulp, which is the edible part of kiwifruits largely used in Chinese traditional medicine, and the lack of the literature on the biological activities of kiwifruits peel, the aim of this work was to investigate the *in vitro* anti-inflammatory activity of a peel extract rich in polyphenols obtained from *Actinidia deliciosa* on activated human THP-1 monocytes, through the analysis of its capability to target multiple processes involved in monocyte-mediated inflammation. Indeed, we investigated the capability of the kiwifruit peel extract to inhibit the production of a number of inflammatory mediators including interleukins (IL-6, IL-1β) and tumor necrosis factor (TNF-α) pro-inflammatory cytokines, high mobility group box 1 (HMGB1) danger signal and granzyme B serine protease, to prevent the activation of STAT3 signaling and to promote autophagy.

2. Material and methods

2.1. Plant material

Low-weight fruits *Actinidia deliciosa* (A.Chev.) C.F.Liang & A.R.Ferguson*,* cv Hayward were collected from Stefano Della Bianca orchard in Cisterna di Latina (Latina, Italy; Latitude: 41°31'42.8"N; Longitude: 12°47'31.3"E) in July 2016. A representative of the cultivar has been

transplanted in the “Orto dei semplici” section of the “Angelo Rambelli” Botanical Garden of the University of Tuscia, under the accession GS159.

2.2. Kiwifruit peel extract preparation and characterization

A kiwifruit peel extract rich in polyphenols was prepared according to a procedure already optimized in our laboratory (Pinelli et al., 2013). Briefly, the peel was isolated from fruits, weighed, frozen in liquid nitrogen and crushed in a mortar. 1 g of the resulting powder was extracted with 50 mL of ethanol/ water=70/30 v/v (water at pH 3.2) under magnetic stirring at room temperature for 24 h. After centrifugation of the mixture, the supernatant was recovered and stored at -20 °C.
The quali-quantitative characterization of polyphenols present in the extract was carried out by HPLC-DAD-ESI-MS using a HP-1100 liquid chromatograph equipped with a DAD detector and a HP 1100 MSD API-electrospray (Agilent Technologies) operating both in negative and positive ionization mode and a column Zorbax SB-Aq 4.6 mm ID x 150 mm (5 µm, Agilent) according to already reported in our laboratory (Pinelli et al., 2013). Polyphenols were identified by retention times, spectroscopic and MS data. Procyanidins and hydroxycinnamic acid derivatives were quantified at 280 and 330 nm using catechin and caffeic acid as reference standard, respectively. The quantitative determination was carried out using five-point regression curves (with r^2>0.9998). All determinations were carried out in triplicate.

Results are given as means and expressed as mg per gram of peel (Table 1) considering that, each mL of extract is referred to 0.02 g of kiwifruit peel; standard error was always <5% (Pinelli et al., 2013).

For the biological activities, a stock solution of the extract solubilized in ethanol/water=70/30, corresponding to a concentration of 12 mg/mL, was expressly prepared as briefly described. From a sample of extract solution, the solvent was removed under reduced pressure by a rotary evaporator (Heidolph, Germany) to afford a powder that was weighted and solubilized with a known volume of ethanol/water=70/30 to obtain 12 mg/mL (stock solution). Then, cells were incubated with the indicated concentrations of the extract (25, 50, 100 and 200 µg/mL), by diluting the stock solution in the cell culture medium. The final concentration of ethanol in the cell culture medium, for both test and corresponding control experiment, was always <1.6 %, compatible with cell viability (assessed by the trypan blue dye exclusion assay) and function (assessed by the analysis of cell proliferation).

2.3. Cell culture

THP-1 human monocytic cells, derived from a patient with acute monocytic leukemia (AML), from American Type Culture Collection (ATCC® TIB-202™; ATCC, MD, USA), were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% FCS (HyClone, Logan, UT, USA), 100 µg/ml of streptomycin and 100 IU/ml penicillin; they were maintained in a 5% CO_2 incubator at 37°C. Cells were mycoplasma free (EZ-PCR Mycoplasma test kit; Biological Industries, Cromwell, CT, USA).

2.4. Cell treatments

For THP-1 activation, cells were cultured with ~~lipopolysaccharide (LPS, 100 ng/mL, Sigma-Aldrich)~~ 100 ng/mL lipopolysaccharide (LPS) from Escherichia coli O55:B5 (L2880 Sigma) for 24 h~~. THP-1 cells were treated with the extract or the ethanolic solution (Control)~~ THP-1 cells were incubated with ethanolic solution alone (Control) or with the extract, solubilized in ethanolic solution, at the indicated doses, for the indicated times. For the autophagic investigation, THP-1 cells were cultured with the extract (50 µg/mL) for 24 h and then treated with Bafilomycin A1 (Baf) (20 nM) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), an inhibitor of vacuolar-H+-ATPase, for the last 2 h. In some experiments, 3-methyladenine (3-MA) (0.2 mM) (Santa Cruz Biotechnology Inc.) was added 2 h before the extract treatment.

2.5. Antibodies

The following primary antibodies were used: rabbit polyclonal anti-IL-6 (Gene Tex, Alton Pkwy Irvine, CA), rabbit polyclonal anti-IL-1β (Gene Tex, CA, USA), mouse monoclonal anti-TNFα (Santa Cruz), rabbit polyclonal anti-HMGB1 (Abcam, Cambridge, UK), mouse monoclonal anti-granzyme B (Calbiochem, San Diego, CA, USA), mouse monoclonal anti-phospho-STAT3 (pY705) (BD Transduction Laboratories, San Jose, CA, USA), mouse monoclonal anti-STAT3 (BD Transduction Laboratories), rabbit polyclonal anti-LC3 (Novus Biologicals, Littleton, CO, USA), mouse monoclonal anti-p62 (BD Transduction Laboratories) and mouse monoclonal anti-β-actin Ac-40 (Sigma-Aldrich).

2.6. Cell viability and proliferation assay

After each treatment, cell viability was assessed by the trypan blue dye exclusion assay, as previously reported (D'Eliseo et al., 2017). The analysis of cell proliferation was performed using 0.05% trypan blue solution to count the number of live cells within a Neubauer chamber. At least three replicate counts were conducted by the same operator at each time.

2.7 Cytokine measurement

Cytokines were measured in conditioned media using the MILLIPLEX® MAP Human Cytokine/Chemokine Magnetic Bead panel (Millipore, Billerica, MA, USA) according to the manufacturer's instructions and detected by Bio-Plex® MAGPIX™ Multiplex Reader (Bio-rad).

2.8. Western blot analysis

Cells were washed twice in PBS and cell lysates were prepared by a solution containing 50 mM TRIS-HCl pH 7.6, 150 mM NaCl, 0.5% TRITON X-100, 0.5% Sodium deoxycolate, 0.1% SDS and the protease inhibitor mixture ‘‘Complete’’ (Roche Diagnostic GmbH, Mannheim, Germany). For the experiments analyzing the extracellular release of inflammatory mediators, the conditioned media was collected. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes (Whatman-Protan, Sigma-Aldrich). The membranes were blocked with 5% Non-Fat Dry Milk (Bio-Rad, Hercules, CA, USA), probed with specific primary antibodies overnight, at 4°C, washed and incubated with appropriated secondary antibodies. The reaction was revealed by horseradish peroxidase (HRP)-coupled secondary reagents (Bio-Rad) and developed by enhanced chemiluminescence (Amersham ECL Western Blotting Detection Reagent, GE Healthcare, MI, Italy).

2.9. Densitometric analysis

The quantification of protein bands was performed by densitometric analysis using Quantity One 1-D analysis software (Bio-Rad) and band intensities (b.i., band volume/area x mean pixel intensity), normalized for β-actin, as previously reported (D'Eliseo et al., 2017).

2.10. Statistical analysis

Student’s *t* test was applied for all analyses; $p<0.05$ was considered significant. All experiments were performed at least three times.

3. Results and discussion

3.1. Kiwifruit peel extract preparation and characterization

The peel of the green berry of *Actinidia deliciosa* (A.Chev.) C.F.Liang & A.R.Ferguson, cv Hayward was treated with ethanol/acidified water (pH 3.2)=70/30 v/v according to a procedure already optimized in our laboratory (Pinelli et al., 2013) to obtain a polyphenolic-rich extract. The quali-quantitative analysis of the extract was performed by HPLC-DAD-ESI-MS. As reported in Table 1, procyanidins dimers, trimers and tetramers resulted the main class of polyphenols present in the extract with 5.422±0.209 mg per gram of peel, representing the 92% w/w of the total;

hydroxycinnamic acid derivatives covered the remaining percentage (8% w/w) with 0.459±0.015 mg/g.

3.2. Effects of kiwifruit peel extract on THP-1 cell viability and proliferation

Kiwifruit peel extract was tested for cytotoxicity in THP-1 cells by culturing them in the presence of increasing extract concentrations (from 12.5 to 200 μg/mL) for 24 h. The trypan-blue dye exclusion assay showed that the extract did not affect the viability of THP-1 cells at none of the concentrations tested (Fig. 1A). Moreover, the evaluation of cell growth showed that the cell number was not affected up to an extract concentration of 100 μg/mL and then decreased in a dose dependent manner (Fig. 1B), suggesting that a proliferative arrest might occur at high concentrations. Thus, all subsequent experiments were performed treating THP-1 cells with a dose of 50 μg/mL for 24 h.

3.3. Kiwifruit peel extract inhibited LPS-inducible and constitutive IL-6, IL-1β and TNF-α production

Pro-inflammatory cytokines IL-6, IL-1β and TNF-α play a pivotal role in the progression of the inflammatory process as a result of monocyte activation. We, therefore, investigated the capability of the extract to affect the production of the three cytokines by activated monocytes. To this purpose, LPS-stimulated and unstimulated (Control) THP-1 cells were treated with the extract and the protein levels of IL-6, IL-1β and TNF-α were measured by Western blot analysis and normalized for β-actin or Ponceau stain. Since it has been previously reported that the maximum of TNF-α release is at earlier times compared to IL-6 and IL-1β, we analyzed the release of TNF-α at 4 h, whereas IL-6 and IL-1β releases were measured at 24 h after treatment. Basal levels of both intracellular and extracellular cytokines were detected in unstimulated THP-1 cells, and, as expected, they increased when THP-1 cells were stimulated with LPS (100 ng/mL) (Fig. 2A and 2B). However, when the cell cultures were treated with the extract, an almost complete reduction of the intracellular content (Fig. 2A) associated to a significant decrease of the release (Fig. 2B) of all the three cytokines was observed in both LPS-stimulated and unstimulated THP-1 cells. Moreover, the inhibition of monocyte-mediated release of cytokines by the extract was confirmed by Luminex multiplex assay (Fig. 2C). These results showed that the kiwifruit peel extract suppressed the production of pro-inflammatory cytokines, indicating a potential anti-inflammatory activity by the extract on inflammatory monocytes.

3.4. Kiwifruit peel extract inhibited LPS-inducible and constitutive HMGB1 production

Extracellular HMGB1 release by the inflammatory microenvironment is involved in the pathogenesis of multiple human inflammatory diseases (e.g., trauma, ischemia, chronic inflammatory disorders, autoimmune diseases and cancer), representing a key molecular target in these diseases (Bianchi et al., 2007). Thus, we investigated the capability of the extract to modulate HMGB1 production by activated monocytes. We found that both the intracellular content (Fig. 2A) and the extracellular release (Fig. 2B, right panel) of HMGB1 were significantly inhibited by the extract in both LPS-stimulated and unstimulated THP-1 cells. This finding further supports the potential of this extract for anti-inflammatory activity and, to the best of our knowledge, this activity has never been described before in studies investigating the anti-inflammatory capability of kiwifruits.

3.5. Kiwifruit peel extract inhibited LPS-inducible and constitutive granzyme B production

The serine protease granzyme B is implicated in extracellular functions involved in inflammation, cytokine activation and autoimmunity, and elevated levels of soluble granzyme B have been reported in plasma of patients with inflammatory diseases (Hiebert et al., 2012). To notice, it has been demonstrated that monocytes/macrophages express granzyme B in the lesion areas of inflammatory diseases, including atherosclerosis and rheumatoid arthritis. Thus, targeting

granzyme B provides new pharmaceutical agents for inflammatory disorders. Therefore, we analyzed whether the extract could affect the production of granzyme B by THP-1 cells. We found that the extract significantly inhibited both the intracellular expression (Fig. 2A) and the extracellular release (Fig. 2B, right panel) of granzyme B by LPS-stimulated and unstimulated THP-1 cells. This is the very first time that a kiwifruit extract has been reported to mediate this activity. This finding further supports the interest in this extract for its anti-inflammatory potential.

3.6. Kiwifruit peel extract prevented STAT3 activation

STAT3 activation by pathogens, growth factors or cytokines, such as IL-6, induces the production of pro-inflammatory cytokines and regulates diverse inflammatory processes, cell proliferation and survival (Yu et al. 2009). In particular, STAT3 activation in monocytes contributes to neovascular age-related macular degeneration (Chen et al., 2016), as well as, promotes liver tumorigenesis (Wu et al., 2011). Indeed, STAT3 in inflammatory monocytes appears a promising target for pharmacological intervention in the prevention and treatment of the development of inflammatory diseases and cancer (Yu et al., 2009). Thus, we examined the capability of the extract to modulate STAT3 activation, by analyzing its phosphorylation at Tyr 705. As expected, STAT3 phosphorylation (p-STAT3) levels significantly increased when THP-1 cells were exposed to LPS (Fig. 3). However, treatment with the extract significantly inhibited p-STAT3 in LPS-stimulated as well as in unstimulated THP-1 cells (Fig. 3). Moreover, considering that STAT3 signaling is triggered by IL-6 upon interaction with IL-6 receptor, we can suppose that a correlation exists between the inhibition of IL-6 production (Fig. 2) and the inhibition of STAT3 activation (Fig. 3). Inhibition of IL-6 production and STAT3 activation by the extract are both relevant findings, since numerous studies demonstrated the association of down-regulation of IL-6 and/or IL-6 signaling (*i.e.,* STAT3) with therapeutic results in inflammatory diseases. In addition, an important role of STAT3 has been identified in the development of different cancers, including AML (Redell et al., 2011). Therefore, our result, showing the inhibition of the constitutive level of STAT3 in unstimulated THP-1 monocytic leukemia cells, suggests that the extract might also exert anti-cancer activity in AML. Considering all these findings, we think that the extract might represent a potential promising candidate to target the IL-6/STAT3 pathway in inflammatory diseases and cancer.

3.7. Kiwifruit peel extract promoted autophagy

Autophagy mediates the clearance of cytoplasmic molecules, organelles and pathogens, and, interestingly, orchestrates inflammation, immunity and cancer (Levine et al., 2011). In particular, activation of autophagy in monocytes exerts anti-inflammatory activity, affecting inflammatory cytokine production by different mechanisms, including the inhibition of inflammasomes, leading to the suppression of IL-1β and IL-18 maturation. Therefore, we examined whether the kiwifruit peel extract could affect autophagy in THP-1 cells. To investigate the autophagic process, two main autophagic markers such as the microtubule-associated protein 1 light chain 3 (LC3) and the sequestosome-1 (SQSTM1)/ubiquitin-binding protein p62 were evaluated by Western blot (Klionsky et al., 2016; D'Eliseo et al., 2017). During autophagy, LC3 is processed post-translationally into soluble LC3-I and, in turn, converted to membrane-bound LC3-II that correlates with the extent of autophagosomes. Therefore, we identified LC3 in the LC3-II form in THP-1 cells and, as shown in Fig. 4, LC3II formation significantly increased after treatment with the extract, suggesting induction of autophagy. Moreover, we observed a further accumulation of LC3-II when extract-treated cells were cultured in the presence of the vacuolar H+-ATPase inhibitor bafilomycin A1 (Baf), confirming the induction of a complete autophagic flux by the extract (Fig. 4). Furthermore, the addition of 3-MA autophagic inhibitor to extract-treated THP-1 cells decreased LC3II formation, confirming thus that LC3II protein levels were the result of the autophagic process (Fig. 4). Then, we analyzed the other autophagic marker such as SQSTM1/p62, which, being part of the assembled autophagosome subsequently degraded in autolysosomes, serves as an

index of autophagic degradation. As shown in Fig. 4, SQSTM1/p62 levels decresed in THP-1 cells treated with the extract, further indicating that the extract was able to promote a complete autophagic process. This is a very significant finding, since defects in autophagy have been linked to a wide range of diseases (e.g., cardiomyopathy, neurodegenerative disorders, cancers), and compounds capable of inducing autophagy have been recently received attention for the treatment of inflammatory diseases (Levine et al., 2011).

4. Conclusions

In summary, ~~as known for pulp and peel extracts from several fruits used in traditional medicine,~~ this study has proved for the first time that an extract rich in procyanidins, obtained from the peel of kiwifruits, is a suppressor of the production of a number of inflammatory mediators such as pro-inflammatory cytokines, HMGB1, granzyme B and STAT3 and a promoter of autophagy on activated human THP-1 monocytes. These results are consistent with reports demonstrating that polyphenols from different origin are endowed with the inhibition of pro-inflammatory cytokine production (Rosillo et al., 2014), the suppression of STAT3 activation (Andujar et al., 2011) and the promotion of autophagy (Pietrocola et al., 2012). All ~~finding~~ the data show that the extract may represent a potential promising anti-inflammatory ~~as well as anti-cancer~~ natural ingredient for pharmaceutical and nutraceutical formulations. However, this assumption requires further verification using *in vivo* models. ~~However, future studies are warranted to investigate the mechanisms underlying the activities exerted by the extract~~

Author contribution

Donatella D'Eliseo, Elisa Pannucci, and Margherita Campo were involved in experimental execution and data collection. Roberta Bernini, Annalisa Romani, Luca Santi and Francesca Velotti were involved in experimental design, in data analysis and interpretation, in manuscript writing and editing.

List of abbreviations

HPLC, high performance liquid chromatography; LPS, lipopolysaccharide; AML, acute monocytic leukemia; IL, interleukin; TNF-α, tumor necrosis factor-α; HMGB1, high-mobility group box 1; STAT3, signal transducer and activator of transcription 3; LC3, protein 1 light chain 3; SQSTM1, sequestosome-1; Baf, bafilomycin A1; DAMPs, damage associated molecular patterns; 3-MA, 3-methyladenine.

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Conflict of interest

No conflict of interests for all authors.

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Table 1. Quali-quantitative analysis of phenolic compounds found in the kiwifruit peel extract.

Compound	t_R (min)	MW	Negative ions (m/z)[a]	λ_{max}, λ_{min}	mg/g [b]
6-Hydroxy-7-(β-D-glucopiranolyloxy coumarin	10.9	340	339, 177		Traces
Caffeic acid derivative	14.0	356	355, 297, 239, 179, 161, 135	326, 264	0.243 ± 0.007
Ferulic acid glucoside	16.2	356	355, 193	312, 255	0.174 ± 0.006
Ferulic acid dehydrodimer	17.5	370	369, 193, 179, 135	324, 265	0.042 ± 0.002
Dimethyl caffeic acid hexoside	16.9	370	369, 207, 191		Traces
Procyanidin trimer	17.8	866	865, 577, 289	280, 258	1.193 ± 0.042
Procyanidin trimer	20.2	866	865, 577, 289	280, 258	0.659 ± 0.025
Procyanidin trimer	21.8	866	865, 577, 289	280, 258	0.665 ± 0.019
Procyanidin tetramer	24.2	1154	1153, 865, 577, 289	280, 258	0.142 ± 0.003
Procyanidin trimer	26.1	866	865, 577, 289	280, 258	0.880 ± 0.035
Procyanidin trimer	27.1	866	865, 577, 289	280, 258	1.187 ± 0.047
Procyanidin dimer	39.9	578	577, 289	280, 258	0.696 ± 0.038
				Total hydroxycinnamic derivatives	0.459 ± 0.015
				Total procyanidins	5.422 ± 0.209
				Total polyphenols	5.881 ± 0.224

[a] *m/z* of the most abundant ESI/MS signals (fragmentor: 120 eV); [b] mg of phenolic compound per gram of peel.

Figure captions

Figure 1. Effects of kiwifruit peel extract on THP-1 cell viability (a) and proliferation (b) at 24 h of treatment. Data show mean of the percentage (%) plus S.D. of three independent experiments; *****p< 0.005.

Figure 2. Effects of kiwifruit peel extract on the intracellular content (a) and the extracellular release (b, c) of inflammatory mediators by THP-1 cells**.** Data show Western blot on cell lysates (a) and conditioned media (b); β-Actin and Ponceau staining served as intracellular and extracellular loading control, respectively; numbers indicate band intensities (b.i.)= band volume/area x mean pixel intensity, normalized for β-actin or Ponceau staining and quantified using Quantity One 1-D analysis software; representative experiments out of three. Histograms represent the mean plus S.D. of cytokine concentrations of three independent experiments, evaluated in conditioned media by Luminex multiplex assay, (c); *****p< 0.005.

Figure 3. Effect of kiwifruit peel extract on STAT3 activation in THP-1 cells. Data show STAT3 Tyr705 phosphorylation (p-STAT3), evaluated by western blot (left panel); total STAT3 and β-actin served as controls; numbers indicate band intensities (b.i.)= band volume/area x mean pixel intensity, normalized for β-actin and quantified using Quantity One 1-D analysis software; representative experiment out of three. Histograms represent the percentage (%), respect to the control, of the mean plus S.D. of the densitometric analysis of p-STAT3/β-actin ratio of three different experiments (right panel); *****p< 0.005.

Figure 4. Effect of kiwifruit peel extract on autophagy in THP-1 cells. Data show the expression of LC3I/II and SQSTM1/p62 autophagic markers, analyzed by western blot (left panel); β-actin was included as loading control; numbers indicate band intensities (b.i.) = band volume/area x mean pixel intensity, normalized for β-actin and quantified using Quantity One 1-D analysis software; representative experiment out of three. Histograms represent the mean plus S.D. of the densitometric analysis of LC3II/β-actin and p62/β-actin ratio of three independent experiments (right panel); *****p< 0.005.

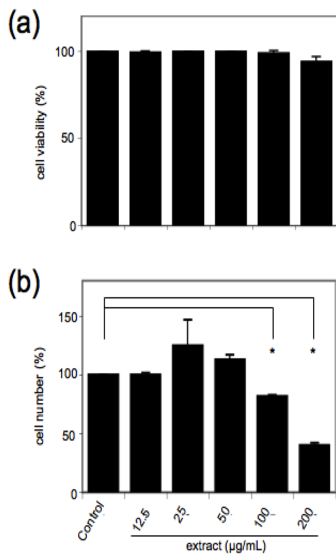


Figure 1

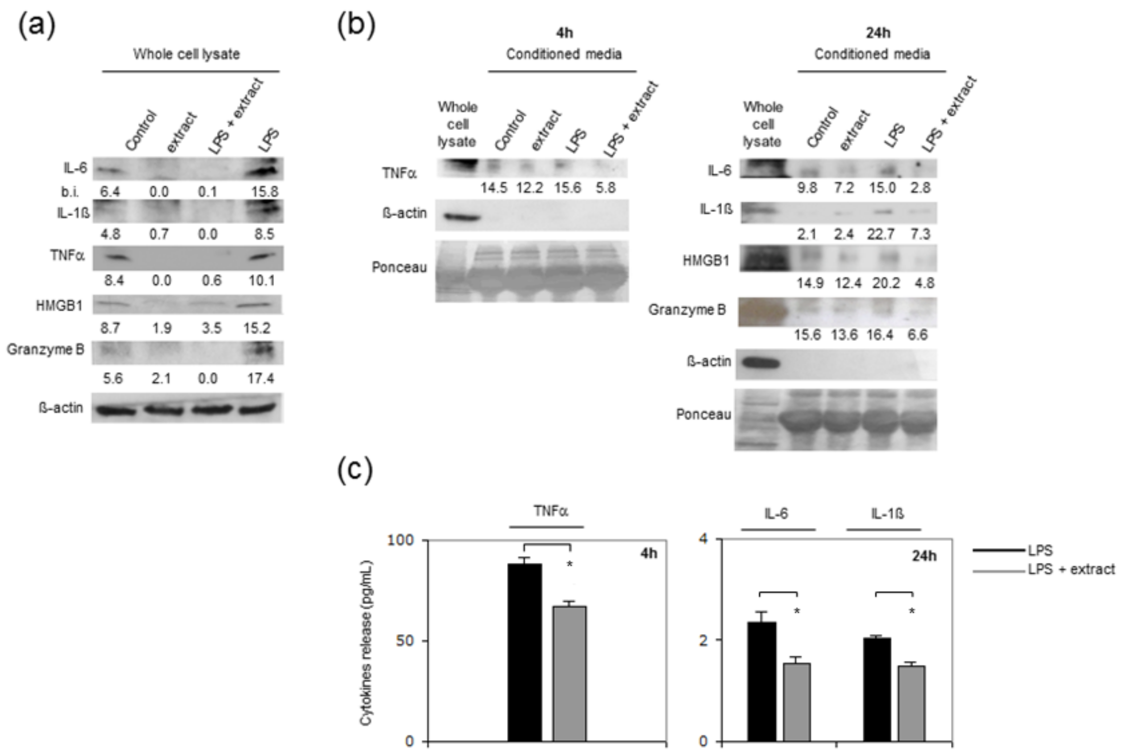


Figure 2.

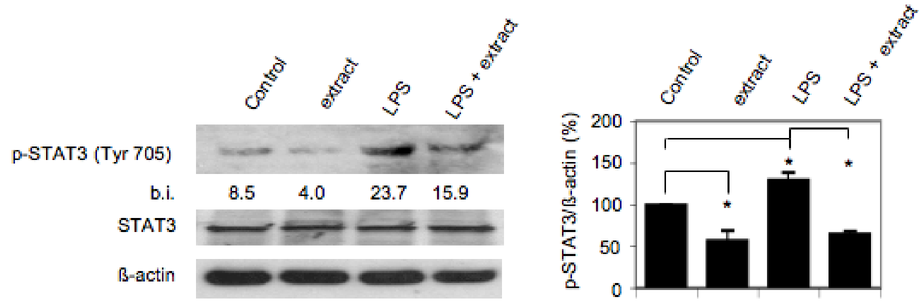


Figure 3

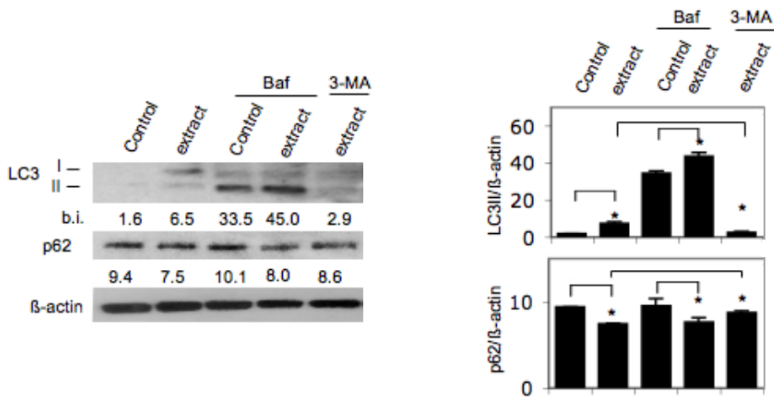
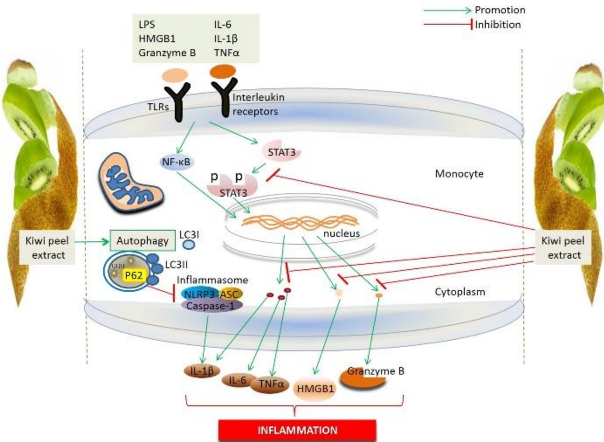


Figure 4

Graphical abstract

In vitro studies on anti-inflammatory activities of kiwifruit peel extract in human THP-1 monocytes



In vitro studies on anti-inflammatory activities of kiwifruit peel extract in human THP-1 monocytes

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Abstract
Ethnopharmacological relevance: Kiwifruit is native to eastern China and many are the references about the consumption of fruits and fruits extracts of the *Actinidia* plants in Chinese traditional medicine as therapeutic food supplements to prevent and/or counteract numerous disorders including inflammation-related diseases like cancer.
Aim of the study: Aim of the present work was to obtain a kiwifruit peel extract, rich in polyphenols, and to explore the anti-inflammatory potential by analyzing its capability to target multiple pathways involved in monocyte-mediated inflammatory response.
Materials and Methods: The extract was obtained from the fruit peel of *Actinidia deliciosa* (A.Chev.) C.F.Liang & A.R.Ferguson, cv Hayward and characterized by HPLC-DAD-ESI-MS. Lipopolysaccharide-stimulated THP-1 monocytes were used as a model of human inflammation *in vitro*.
Results: Analytical data evidenced that procyanidins resulted the main polyphenols present in the extract, representing the 92% w/w of the total. The extract inhibited the production of inflammatory molecules such as IL-6, IL-1β, TNF-α pro-inflammatory cytokines, HMGB1 danger signal and granzyme B serine protease by activated monocytes. In particular, an inhibitory activity of 81%, 68%, 63%, 76% and 60% on the extracellular release of IL-6, IL-1β, TNF-α, HMGB1 and granzyme B, respectively, was observed by western blot analysis. Moreover, the extract prevented STAT3 activation and promoted autophagy.
Conclusions: The reported findings demonstrated a strong and broad anti-inflammatory profile of the kiwifruit peel extract, which makes it a promising preventive and therapeutic natural ingredient for nutraceutical, cosmetic and pharmaceutical formulations to counteract multiple inflammatory disorders.

Keywords: kiwifruit (*Actinidia deliciosa,* cv Hayward) peel extract, procyanidins, inflammation, STAT3, autophagy.

1. Introduction

Kiwifruit is the edible berry of the *Actinidia* plant (Actinidiaceae). The most commonly consumed is the green berry of the *Actinidia deliciosa* (A.Chev.) C.F.Liang & A.R.Ferguson, cv Hayward. According to data provided by the Food and Agriculture Organization (FAO) of the United States, kiwifruits cultivation is relevant in China (the native country), Italy, New Zealand, Chile and Greece, which represent the five largest producers in the world.

The use of kiwifruit in Chinese traditional medicine is well documented since the 1400's A.D. while the first recorded reference on the effects against a "cancer-like" disease dates even back to the 720's B.C. (Motohashi et al., 2002; Singletary, K., 2012; Sun et al., 2012). Over the years, kiwifruit has known an ever-wider use in traditional medicine to prevent cardiovascular and degenerative diseases, to relieve disorders caused from dyspepsia, rheumatism, digestive problems and to treat stomach, liver and rectal cancer (Zhi, 1980; Yang, 1981).

More recently, several studies have been focused on the biological activities of the kiwifruit, by individually analyzing extracts derived from either the fruit (Motohashi et al., 2002; Hunter et al., 2011) or the peel (Motohashi et al., 2001; Yang et al., 2013; Lee et al., 2014). However, to the best of our knowledge, the anti-inflammatory effects of kiwifruit peel extract have never been investigated so far.

Inflammation is the body's normal response to injuries or infections, but, when excessive or persistent, it causes and advances many serious inflammatory diseases including cancer (Lansky et al., 2007; Cragg et al., 2009; Martinez-Micaelo et al., 2012; Bernini et al., 2013 and 2015). Monocytes and monocyte-derived cells represent critical players in the orchestration of the inflammatory response, representing thus key therapeutic targets for disease treatment. Several studies reported that polyphenols (Andujar et al., 2011; Pietrocola et al., 2012; Bernini et al., 2013 and 2015; Rosillo et al., 2014; Liu et al., 2018), and, in particular, procyanidins exerted an anti-inflammatory activity (Bak et al., 2013; De La Iglesia et al., 2010; Martinez-Micaelo et al., 2012).

Based on the consideration that the peel is intimately connected to the pulp, which is the edible part of kiwifruits largely used in Chinese traditional medicine, and the lack of the literature on the biological activities of kiwifruits peel, the aim of this work was to investigate the *in vitro* anti-inflammatory activity of a peel extract rich in polyphenols obtained from *Actinidia deliciosa* on activated human THP-1 monocytes, through the analysis of its capability to target multiple processes involved in monocyte-mediated inflammation. Indeed, we investigated the capability of the kiwifruit peel extract to inhibit the production of a number of inflammatory mediators including interleukins (IL-6, IL-1β) and tumor necrosis factor (TNF-α) pro-inflammatory cytokines, high mobility group box 1 (HMGB1) danger signal and granzyme B serine protease, to prevent the activation of STAT3 signaling and to promote autophagy.

2. Material and methods

2.1. Plant material

Low-weight fruits *Actinidia deliciosa* (A.Chev.) C.F.Liang & A.R.Ferguson, cv Hayward were collected from Stefano Della Bianca orchard in Cisterna di Latina (Latina, Italy; Latitude: 41°31'42.8"N; Longitude: 12°47'31.3"E) in July 2016. A representative of the cultivar has been transplanted in the "Orto dei semplici" section of the "Angelo Rambelli" Botanical Garden of the University of Tuscia, under the accession GS159.

2.2. Kiwifruit peel extract preparation and characterization

A kiwifruit peel extract rich in polyphenols was prepared according to a procedure already optimized in our laboratory (Pinelli et al., 2013). Briefly, the peel was isolated from fruits, weighed, frozen in liquid nitrogen and crushed in a mortar. 1 g of the resulting powder was extracted with 50 mL of ethanol/ water=70/30 v/v (water at pH 3.2) under magnetic stirring at room temperature for 24 h. After centrifugation of the mixture, the supernatant was recovered and stored at -20 °C.

The quali-quantitative characterization of polyphenols present in the extract was carried out by HPLC-DAD-ESI-MS using a HP-1100 liquid chromatograph equipped with a DAD detector and a HP 1100 MSD API-electrospray (Agilent Technologies) operating both in negative and positive ionization mode and a column Zorbax SB-Aq 4.6 mm ID x 150 mm (5 µm, Agilent) according to already reported in our laboratory (Pinelli et al., 2013). Polyphenols were identified by retention times, spectroscopic and MS data. Procyanidins and hydroxycinnamic acid derivatives were quantified at 280 and 330 nm using catechin and caffeic acid as reference standard, respectively. The quantitative determination was carried out using five-point regression curves (with r^2>0.9998). All determinations were carried out in triplicate.

Results are given as means and expressed as mg per gram of peel (Table 1) considering that, each mL of extract is referred to 0.02 g of kiwifruit peel; standard error was always <5% (Pinelli et al., 2013).

For the biological activities, a stock solution of the extract solubilized in ethanol/water=70/30, corresponding to a concentration of 12 mg/mL, was expressly prepared as briefly described. From a sample of extract solution, the solvent was removed under reduced pressure by a rotary evaporator (Heidolph, Germany) to afford a powder that was weighted and solubilized with a known volume of ethanol/water=70/30 to obtain 12 mg/mL (stock solution). Then, cells were incubated with the indicated concentrations of the extract (25, 50, 100 and 200 µg/mL), by diluting the stock solution in the cell culture medium. The final concentration of ethanol in the cell culture medium, for both test and corresponding control experiment, was always <1.6 %, compatible with cell viability (assessed by the trypan blue dye exclusion assay) and function (assessed by the analysis of cell proliferation).

2.3. Cell culture

THP-1 human monocytic cells, derived from a patient with acute monocytic leukemia (AML), from American Type Culture Collection (ATCC® TIB-202™; ATCC, MD, USA), were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% FCS (HyClone, Logan, UT, USA), 100 µg/ml of streptomycin and 100 IU/ml penicillin; they were maintained in a 5% CO_2 incubator at 37°C. Cells were mycoplasma free (EZ-PCR Mycoplasma test kit; Biological Industries, Cromwell, CT, USA).

2.4. Cell treatments

For THP-1 activation, cells were cultured with 100 ng/mL lipopolysaccharide (LPS) from Escherichia coli O55:B5 (L2880 Sigma) for 24 h. THP-1 cells were incubated with ethanolic solution alone (Control) or with the extract, solubilized in ethanolic solution, at the indicated doses, for the indicated times. For the autophagic investigation, THP-1 cells were cultured with the extract (50 µg/mL) for 24 h and then treated with Bafilomycin A1 (Baf) (20 nM) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), an inhibitor of vacuolar-H+-ATPase, for the last 2 h. In some experiments, 3-methyladenine (3-MA) (0.2 mM) (Santa Cruz Biotechnology Inc.) was added 2 h before the extract treatment.

2.5. Antibodies

The following primary antibodies were used: rabbit polyclonal anti-IL-6 (Gene Tex, Alton Pkwy Irvine, CA), rabbit polyclonal anti-IL-1β (Gene Tex, CA, USA), mouse monoclonal anti-TNFα (Santa Cruz), rabbit polyclonal anti-HMGB1 (Abcam, Cambridge, UK), mouse monoclonal anti-granzyme B (Calbiochem, San Diego, CA, USA), mouse monoclonal anti-phospho-STAT3 (pY705) (BD Transduction Laboratories, San Jose, CA, USA), mouse monoclonal anti-STAT3 (BD Transduction Laboratories), rabbit polyclonal anti-LC3 (Novus Biologicals, Littleton, CO, USA), mouse monoclonal anti-p62 (BD Transduction Laboratories) and mouse monoclonal anti-β-actin Ac-40 (Sigma-Aldrich).

2.6. Cell viability and proliferation assay

After each treatment, cell viability was assessed by the trypan blue dye exclusion assay, as previously reported (D'Eliseo et al., 2017). The analysis of cell proliferation was performed using 0.05% trypan blue solution to count the number of live cells within a Neubauer chamber. At least three replicate counts were conducted by the same operator at each time.

2.7 Cytokine measurement

Cytokines were measured in conditioned media using the MILLIPLEX® MAP Human Cytokine/Chemokine Magnetic Bead panel (Millipore, Billerica, MA, USA) according to the manufacturer's instructions and detected by Bio-Plex® MAGPIX™ Multiplex Reader (Bio-rad).

2.8. Western blot analysis

Cells were washed twice in PBS and cell lysates were prepared by a solution containing 50 mM TRIS-HCl pH 7.6, 150 mM NaCl, 0.5% TRITON X-100, 0.5% Sodium deoxycolate, 0.1% SDS and the protease inhibitor mixture ‘‘Complete’’ (Roche Diagnostic GmbH, Mannheim, Germany). For the experiments analyzing the extracellular release of inflammatory mediators, the conditioned media was collected. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes (Whatman-Protan, Sigma-Aldrich). The membranes were blocked with 5% Non-Fat Dry Milk (Bio-Rad, Hercules, CA, USA), probed with specific primary antibodies overnight, at 4°C, washed and incubated with appropriated secondary antibodies. The reaction was revealed by horseradish peroxidase (HRP)-coupled secondary reagents (Bio-Rad) and developed by enhanced chemiluminescence (Amersham ECL Western Blotting Detection Reagent, GE Healthcare, MI, Italy).

2.9. Densitometric analysis

The quantification of protein bands was performed by densitometric analysis using Quantity One 1-D analysis software (Bio-Rad) and band intensities (b.i., band volume/area x mean pixel intensity), normalized for β-actin, as previously reported (D'Eliseo et al., 2017).

2.10. Statistical analysis

Student’s *t* test was applied for all analyses; $p<0.05$ was considered significant. All experiments were performed at least three times.

3. Results and discussion

3.1. Kiwifruit peel extract preparation and characterization

The peel of the green berry of *Actinidia deliciosa* (A.Chev.) C.F.Liang & A.R.Ferguson, cv Hayward was treated with ethanol/acidified water (pH 3.2)=70/30 v/v according to a procedure already optimized in our laboratory (Pinelli et al., 2013) to obtain a polyphenolic-rich extract. The quali-quantitative analysis of the extract was performed by HPLC-DAD-ESI-MS. As reported in Table 1, procyanidins dimers, trimers and tetramers resulted the main class of polyphenols present in the extract with 5.422±0.209 mg per gram of peel, representing the 92% w/w of the total; hydroxycinnamic acid derivatives covered the remaining percentage (8% w/w) with 0.459±0.015 mg/g.

3.2. Effects of kiwifruit peel extract on THP-1 cell viability and proliferation

Kiwifruit peel extract was tested for cytotoxicity in THP-1 cells by culturing them in the presence of increasing extract concentrations (from 12.5 to 200 μg/mL) for 24 h. The trypan-blue dye exclusion assay showed that the extract did not affect the viability of THP-1 cells at none of the concentrations tested (Fig. 1A). Moreover, the evaluation of cell growth showed that the cell number was not affected up to an extract concentration of 100 μg/mL and then decreased in a dose dependent manner (Fig. 1B), suggesting that a proliferative arrest might occur at high

concentrations. Thus, all subsequent experiments were performed treating THP-1 cells with a dose of 50 μg/mL for 24 h.

3.3. Kiwifruit peel extract inhibited LPS-inducible and constitutive IL-6, IL-1β and TNF-α production

Pro-inflammatory cytokines IL-6, IL-1β and TNF-α play a pivotal role in the progression of the inflammatory process as a result of monocyte activation. We, therefore, investigated the capability of the extract to affect the production of the three cytokines by activated monocytes. To this purpose, LPS-stimulated and unstimulated (Control) THP-1 cells were treated with the extract and the protein levels of IL-6, IL-1β and TNF-α were measured by Western blot analysis and normalized for β-actin or Ponceau stain. Since it has been previously reported that the maximum of TNF-α release is at earlier times compared to IL-6 and IL-1β, we analyzed the release of TNF-α at 4 h, whereas IL-6 and IL-1β releases were measured at 24 h after treatment. Basal levels of both intracellular and extracellular cytokines were detected in unstimulated THP-1 cells, and, as expected, they increased when THP-1 cells were stimulated with LPS (100 ng/mL) (Fig. 2A and 2B). However, when the cell cultures were treated with the extract, an almost complete reduction of the intracellular content (Fig. 2A) associated to a significant decrease of the release (Fig. 2B) of all the three cytokines was observed in both LPS-stimulated and unstimulated THP-1 cells. Moreover, the inhibition of monocyte-mediated release of cytokines by the extract was confirmed by Luminex multiplex assay (Fig. 2C). These results showed that the kiwifruit peel extract suppressed the production of pro-inflammatory cytokines, indicating a potential anti-inflammatory activity by the extract on inflammatory monocytes.

3.4. Kiwifruit peel extract inhibited LPS-inducible and constitutive HMGB1 production

Extracellular HMGB1 release by the inflammatory microenvironment is involved in the pathogenesis of multiple human inflammatory diseases (e.g., trauma, ischemia, chronic inflammatory disorders, autoimmune diseases and cancer), representing a key molecular target in these diseases (Bianchi et al., 2007). Thus, we investigated the capability of the extract to modulate HMGB1 production by activated monocytes. We found that both the intracellular content (Fig. 2A) and the extracellular release (Fig. 2B, right panel) of HMGB1 were significantly inhibited by the extract in both LPS-stimulated and unstimulated THP-1 cells. This finding further supports the potential of this extract for anti-inflammatory activity and, to the best of our knowledge, this activity has never been described before in studies investigating the anti-inflammatory capability of kiwifruits.

3.5. Kiwifruit peel extract inhibited LPS-inducible and constitutive granzyme B production

The serine protease granzyme B is implicated in extracellular functions involved in inflammation, cytokine activation and autoimmunity, and elevated levels of soluble granzyme B have been reported in plasma of patients with inflammatory diseases (Hiebert et al., 2012). To notice, it has been demonstrated that monocytes/macrophages express granzyme B in the lesion areas of inflammatory diseases, including atherosclerosis and rheumatoid arthritis. Thus, targeting granzyme B provides new pharmaceutical agents for inflammatory disorders. Therefore, we analyzed whether the extract could affect the production of granzyme B by THP-1 cells. We found that the extract significantly inhibited both the intracellular expression (Fig. 2A) and the extracellular release (Fig. 2B, right panel) of granzyme B by LPS-stimulated and unstimulated THP-1 cells. This is the very first time that a kiwifruit extract has been reported to mediate this activity. This finding further supports the interest in this extract for its anti-inflammatory potential.

3.6. Kiwifruit peel extract prevented STAT3 activation

STAT3 activation by pathogens, growth factors or cytokines, such as IL-6, induces the production of pro-inflammatory cytokines and regulates diverse inflammatory processes, cell

proliferation and survival (Yu et al. 2009). In particular, STAT3 activation in monocytes contributes to neovascular age-related macular degeneration (Chen et al., 2016), as well as, promotes liver tumorigenesis (Wu et al., 2011). Indeed, STAT3 in inflammatory monocytes appears a promising target for pharmacological intervention in the prevention and treatment of the development of inflammatory diseases and cancer (Yu et al., 2009). Thus, we examined the capability of the extract to modulate STAT3 activation, by analyzing its phosphorylation at Tyr 705. As expected, STAT3 phosphorylation (p-STAT3) levels significantly increased when THP-1 cells were exposed to LPS (Fig. 3). However, treatment with the extract significantly inhibited p-STAT3 in LPS-stimulated as well as in unstimulated THP-1 cells (Fig. 3). Moreover, considering that STAT3 signaling is triggered by IL-6 upon interaction with IL-6 receptor, we can suppose that a correlation exists between the inhibition of IL-6 production (Fig. 2) and the inhibition of STAT3 activation (Fig. 3). Inhibition of IL-6 production and STAT3 activation by the extract are both relevant findings, since numerous studies demonstrated the association of down-regulation of IL-6 and/or IL-6 signaling (*i.e.,* STAT3) with therapeutic results in inflammatory diseases. In addition, an important role of STAT3 has been identified in the development of different cancers, including AML (Redell et al., 2011). Therefore, our result, showing the inhibition of the constitutive level of STAT3 in unstimulated THP-1 monocytic leukemia cells, suggests that the extract might also exert anti-cancer activity in AML. Considering all these findings, we think that the extract might represent a potential promising candidate to target the IL-6/STAT3 pathway in inflammatory diseases and cancer.

3.7. Kiwifruit peel extract promoted autophagy

Autophagy mediates the clearance of cytoplasmic molecules, organelles and pathogens, and, interestingly, orchestrates inflammation, immunity and cancer (Levine et al., 2011). In particular, activation of autophagy in monocytes exerts anti-inflammatory activity, affecting inflammatory cytokine production by different mechanisms, including the inhibition of inflammasomes, leading to the suppression of IL-1β and IL-18 maturation. Therefore, we examined whether the kiwifruit peel extract could affect autophagy in THP-1 cells. To investigate the autophagic process, two main autophagic markers such as the microtubule-associated protein 1 light chain 3 (LC3) and the sequestosome-1 (SQSTM1)/ubiquitin-binding protein p62 were evaluated by Western blot (Klionsky et al., 2016; D'Eliseo et al., 2017). During autophagy, LC3 is processed post-translationally into soluble LC3-I and, in turn, converted to membrane-bound LC3-II that correlates with the extent of autophagosomes. Therefore, we identified LC3 in the LC3-II form in THP-1 cells and, as shown in Fig. 4, LC3II formation significantly increased after treatment with the extract, suggesting induction of autophagy. Moreover, we observed a further accumulation of LC3-II when extract-treated cells were cultured in the presence of the vacuolar H+-ATPase inhibitor bafilomycin A1 (Baf), confirming the induction of a complete autophagic flux by the extract (Fig. 4). Furthermore, the addition of 3-MA autophagic inhibitor to extract-treated THP-1 cells decreased LC3II formation, confirming thus that LC3II protein levels were the result of the autophagic process (Fig. 4). Then, we analyzed the other autophagic marker such as SQSTM1/p62, which, being part of the assembled autophagosome subsequently degraded in autolysosomes, serves as an index of autophagic degradation. As shown in Fig. 4, SQSTM1/p62 levels decresed in THP-1 cells treated with the extract, further indicating that the extract was able to promote a complete autophagic process. This is a very significant finding, since defects in autophagy have been linked to a wide range of diseases (e.g., cardiomyopathy, neurodegenerative disorders, cancers), and compounds capable of inducing autophagy have been recently received attention for the treatment of inflammatory diseases (Levine et al., 2011).

4. Conclusions

In summary, this study has proved for the first time that an extract rich in procyanidins, obtained from the peel of kiwifruits, is a suppressor of the production of a number of inflammatory mediators

such as pro-inflammatory cytokines, HMGB1, granzyme B and STAT3 and a promoter of autophagy on activated human THP-1 monocytes. These results are consistent with reports demonstrating that polyphenols from different origin are endowed with the inhibition of pro-inflammatory cytokine production (Rosillo et al., 2014), the suppression of STAT3 activation (Andujar et al., 2011) and the promotion of autophagy (Pietrocola et al., 2012). All the data show that the extract may represent a potential promising anti-inflammatory natural ingredient for pharmaceutical and nutraceutical formulations. However, this assumption requires further verification using *in vivo* models.

Author contribution

Donatella D'Eliseo, Elisa Pannucci, and Margherita Campo were involved in experimental execution and data collection. Roberta Bernini, Annalisa Romani, Luca Santi and Francesca Velotti were involved in experimental design, in data analysis and interpretation, in manuscript writing and editing.

List of abbreviations

HPLC, high performance liquid chromatography; LPS, lipopolysaccharide; AML, acute monocytic leukemia; IL, interleukin; TNF-α, tumor necrosis factor-α; HMGB1, high-mobility group box 1; STAT3, signal transducer and activator of transcription 3; LC3, protein 1 light chain 3; SQSTM1, sequestosome-1; Baf, bafilomycin A1; DAMPs, damage associated molecular patterns; 3-MA, 3-methyladenine.

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Conflict of interest

No conflict of interests for all authors.

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Table 1. Quali-quantitative analysis of phenolic compounds found in the kiwifruit peel extract.

Compound	**t_R (min)**	**MW**	**Negative ions (m/z)[a]**	**λ_{max}, λ_{min}**	**mg/g [b]**
6-Hydroxy-7-(β-D-glucopiranolyloxy coumarin	10.9	340	339, 177		Traces
Caffeic acid derivative	14.0	356	355, 297, 239, 179, 161, 135	326, 264	0.243 ± 0.007
Ferulic acid glucoside	16.2	356	355, 193	312, 255	0.174 ± 0.006
Ferulic acid dehydrodimer	17.5	370	369, 193, 179, 135	324, 265	0.042 ± 0.002
Dimethyl caffeic acid hexoside	16.9	370	369, 207, 191		Traces
Procyanidin trimer	17.8	866	865, 577, 289	280, 258	1.193 ± 0.042
Procyanidin trimer	20.2	866	865, 577, 289	280, 258	0.659 ± 0.025
Procyanidin trimer	21.8	866	865, 577, 289	280, 258	0.665 ± 0.019
Procyanidin tetramer	24.2	1154	1153, 865, 577, 289	280, 258	0.142 ± 0.003
Procyanidin trimer	26.1	866	865, 577, 289	280, 258	0.880 ± 0.035
Procyanidin trimer	27.1	866	865, 577, 289	280, 258	1.187 ± 0.047
Procyanidin dimer	39.9	578	577, 289	280, 258	0.696 ± 0.038
				Total hydroxycinnamic derivatives	0.459 ± 0.015
				Total procyanidins	5.422 ± 0.209
				Total polyphenols	5.881 ± 0.224

[a] *m/z* of the most abundant ESI/MS signals (fragmentor: 120 eV); [b] mg of phenolic compound per gram of peel.

Figure captions

Figure 1. Effects of kiwifruit peel extract on THP-1 cell viability (a) and proliferation (b) at 24 h of treatment. Data show mean of the percentage (%) plus S.D. of three independent experiments; *****p< 0.005.

Figure 2. Effects of kiwifruit peel extract on the intracellular content (a) and the extracellular release (b, c) of inflammatory mediators by THP-1 cells**.** Data show Western blot on cell lysates (a) and conditioned media (b); β-Actin and Ponceau staining served as intracellular and extracellular loading control, respectively; numbers indicate band intensities (b.i.)= band volume/area x mean pixel intensity, normalized for β-actin or Ponceau staining and quantified using Quantity One 1-D analysis software; representative experiments out of three. Histograms represent the mean plus S.D. of cytokine concentrations of three independent experiments, evaluated in conditioned media by Luminex multiplex assay, (c); *****p< 0.005.

Figure 3. Effect of kiwifruit peel extract on STAT3 activation in THP-1 cells. Data show STAT3 Tyr705 phosphorylation (p-STAT3), evaluated by western blot (left panel); total STAT3 and β-actin served as controls; numbers indicate band intensities (b.i.)= band volume/area x mean pixel intensity, normalized for β-actin and quantified using Quantity One 1-D analysis software; representative experiment out of three. Histograms represent the percentage (%), respect to the control, of the mean plus S.D. of the densitometric analysis of p-STAT3/β-actin ratio of three different experiments (right panel); *****p< 0.005.

Figure 4. Effect of kiwifruit peel extract on autophagy in THP-1 cells. Data show the expression of LC3I/II and SQSTM1/p62 autophagic markers, analyzed by western blot (left panel); β-actin was included as loading control; numbers indicate band intensities (b.i.) = band volume/area x mean pixel intensity, normalized for β-actin and quantified using Quantity One 1-D analysis software; representative experiment out of three. Histograms represent the mean plus S.D. of the densitometric analysis of LC3II/β-actin and p62/β-actin ratio of three independent experiments (right panel); *****p< 0.005.

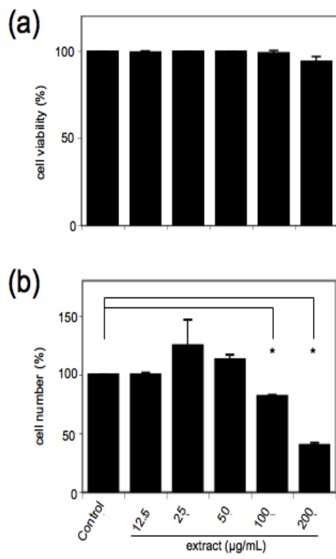

Figure 1

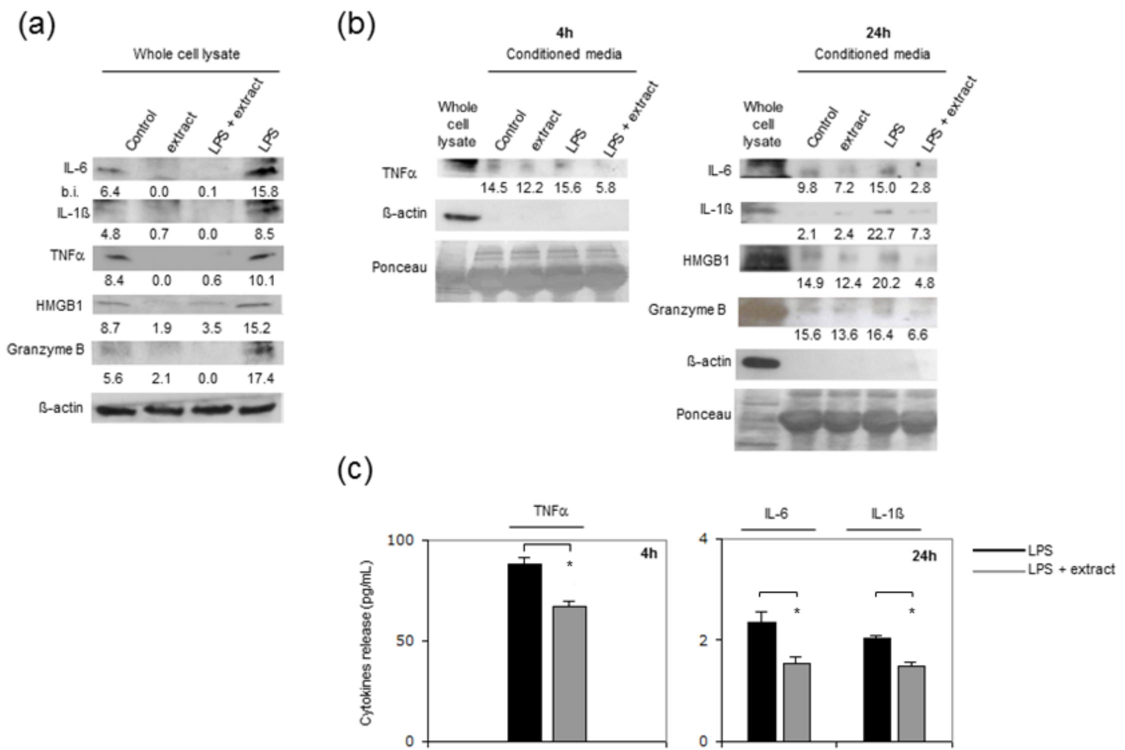

Figure 2.

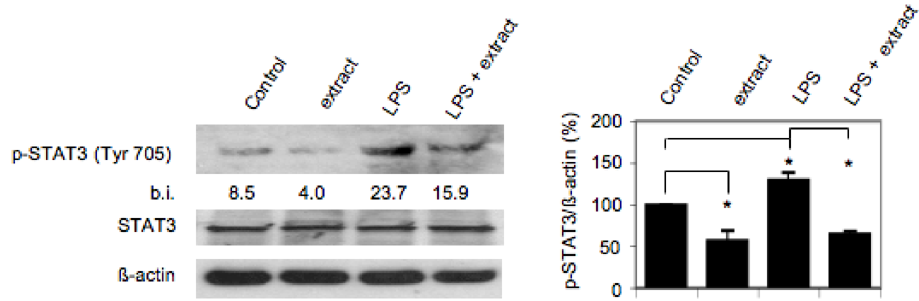

Figure 3

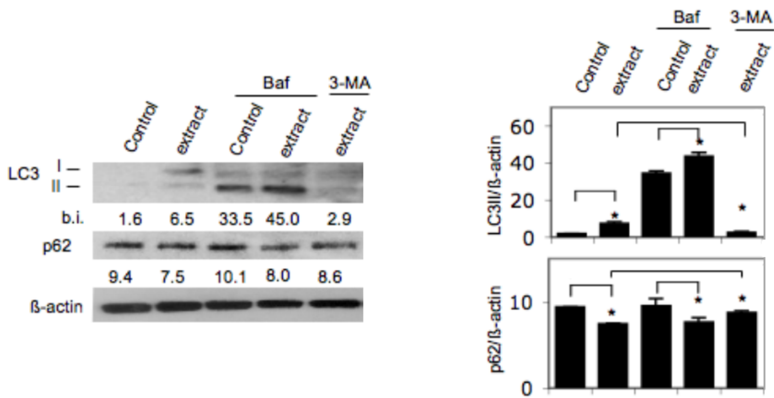

Figure 4