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Junhyo Cho
University of Massachusetts Amherst

Yeonhwa Park
University of Massachusetts Amherst

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Kahweol, a coffee diterpene, increases lifespan via insulin/insulin-like growth factor-1 and AMP-activated protein kinase signaling pathways in *Caenorhabditis elegans*

Junhyo Cho, Yeonhwa Park *

Department of Food Science, University of Massachusetts Amherst, Amherst, MA, 01003, USA

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ABSTRACT

Coffee is one of the most widely consumed beverages and is known to have many health benefits. Our previous study reported that kahweol, a diterpene found in coffee, reduced fat accumulation by reducing food intake in *Caenorhabditis elegans*. Based on the widely known observation of caloric restriction and lifespan, we determined if kahweol extends lifespan in *C. elegans*. Kahweol significantly extended the lifespan of wild-type *C. elegans*. However, kahweol increased the lifespan of the *eat*-2 null mutant that has a reduced food intake phenotype, suggesting that kahweol extends lifespan independent of reduced food intake. Therefore, we further determine the target of kahweol on lifespan extension. Kahweol had no effects on the lifespan of both *daf-2* (the homolog of insulin/insulin-like growth factor-1 receptor) and *daf-16* (the homolog of Forkhead box O transcription factor and a major downstream target of *daf-2*) null mutants, suggesting kahweol extended lifespan via insulin/insulin-like growth factor-1 signaling pathway. In addition, kahweol failed to extend lifespan in *tub-1* (the homolog of TUB bipartite transcription factor) and *aak-2* (the homolog of AMP-activated protein kinase) null mutants, suggesting these roles on kahweol's effect on lifespan. However, the treatment of kahweol increased the lifespan in *sir-2.1* (the homolog of NAD-dependent deacetylase sirtuin-1) and *skn-1* (the homolog of nuclear factor erythroid 2-related factor 2) null mutants over the control, suggesting independent functions of these genes on kahweol's lifespan extension. These results indicate that the insulin/insulin-like growth factor-1 signaling and AMPK pathways may play critical roles in extending lifespan by kahweol in *C. elegans*.

1. Introduction

Coffee is one of the most widely consumed beverages that has many beneficial effects on human health (Cano-Marquina et al., 2013; Farias-Pereira et al., 2018, Farias-Pereira et al., 2020a,b; George et al., 2008; Ludwig et al., 2014; Samoggia and Riedel, 2019). Among various bioactive compounds in coffee, kahweol is a major diterpene that can be consumed at a dose of 0.5 mg and up to 6 mg per cup of coffee, depending on the brewing method (Farias-Pereira et al., 2020b; Gross et al., 1997). Previous studies have reported that the treatment of kahweol has anti-inflammation, anti-tumor, anti-diabetes, anti-oxidation, and anti-adipogenic effects (Farias-Pereira et al., 2020b; Kim et al., 2017; Ren et al., 2019). Although many health-beneficial effects of kahweol have been found, little is known of its potential effects on aging.

Aging is a natural process that affects all living organisms and is characterized by the accumulation of various time-dependent changes in cells and tissues (López-Otín et al., 2013). This progressive aging process is closely linked to developing chronic diseases, including diabetes, cancer, neurodegenerative disorders, and many others (Niccoli and Partridge, 2012). Thus, slowing aging and mitigating age-related degeneration can significantly improve the overall quality of life. However, aging studies are challenging, including determining the underlying mechanisms, due to the long duration needed for experiments (Holtze et al., 2021). To address these challenges, researchers have used *Caenorhabditis elegans* for aging studies.

C. elegans is a eukaryotic invertebrate animal model that can be easily maintained in the laboratory by feeding non-pathogenic *Escherichia coli* OP50 as a standard food source and has a relatively short lifespan, ~1 month at room temperature (Holtze et al., 2021; Shen et al., 2017; Yue et al., 2019). The genetic properties of *C. elegans* are well known and conserve over 65% of homologs that are disease-related genes in humans (Shen et al., 2018a,b). Based on these characteristics,

* Corresponding author.
E-mail addresses: junhyocho@umass.edu (J. Cho), ypark@umass.edu (Y. Park).

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this transparent nematode is widely used in aging studies and various life science research (Kaletta and Hengartner, 2006; Shen et al., 2018a,b; Tissenbaum, 2014).

Our previous study has identified that treatment of kahweol reduces food intake and fat accumulation in *C. elegans* (Farias-Pereira et al., 2020b). Meanwhile, it has been previously reported that caloric restriction leads to lifespan extension (Weindruch and Sohal, 1997). Based on these findings, we hypothesize that kahweol may extend lifespan. Thus, this study aimed to determine the kahweol's effect on lifespan using *C. elegans* and the potential mechanisms of anti-aging properties of kahweol.

2. Materials and methods

2.1. Materials

Kahweol, with purity of ≥98% (CAT#: 14015), was purchased from Cayman Chemical (Ann Arbor, MI, USA). Ampicillin and carbenicillin were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA). All *C. elegans* strains and *E. coli* OP50 were purchased from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN, USA).

2.2. C. elegans maintenance

C. elegans maintenance was done as previously described with slight modifications based on WormBook (Farias-Pereira et al., 2020a,b; Stiernagle, 2006; Yue et al., 2019). *C. elegans* was kept using nematode growth media (NGM) to obtain synchronized worms for experiments. *C. elegans* strains used in this study were: N2 Bristol (wild-type), *daf-2* (CB1370)*III*, *daf-16* (GR1307)*I*, *eat-2* (DA1116)*II*, *sir-2.1* (VC119)*IV*, *aak-2* (RB754)*X*, *skn-1* (GR2245)*IV*, and *tub-1* (DG2179)*II* (Table S1.). Worms have been removed using sodium hydroxide/bleach solution to collect only eggs. Synchronized L1 stage worms have been cultured using liquid nematode growth media (S-complete) containing ampicillin (100 μg/mL) and carbenicillin (50 μg/mL). Worms were fed with live *E. coli* OP50 and incubated at 20 °C until worms reached the L4 stage for further experiments, except the *daf-2* mutant was incubated at 15 °C.

2.3. Lifespan assay

L4 stage worms were transferred to HTS Transwell®-96 well permeable plates (Corning Inc, NY, USA). In our previous publication, the Transwell plate was first suggested for lifespan assay over NGM plates for an easier and more efficient media exchange (Shen et al., 2017). A final concentration of 120 μM of 2′-deoxy-5-fluorouridine (FUDR) was added to S-complete to inhibit eggs from hatching starting from the L4 stage. Kahweol was dissolved in dimethyl sulfoxide (DMSO), and a final concentration range of 10–100 μM of kahweol was used. The dose range was chosen based on our previous publication (Farias-Pereira et al., 2020b). All treatments and controls had a final concentration of 0.1% DMSO. The fresh media and *E. coli* OP50 were provided every two days using a Transwell plate. Initial treatment for adult stage worms was defined as day 0 of adult age, and the survival score of worms was recorded every two days until all worms died. Each treatment group has approximately 100–120 worms.

2.4. Reactive oxygen species (ROS) levels

Measurement of intracellular reactive oxygen species (ROS) levels using 2′,7′-dichlorofluorescein diacetate (H2DCF-DA) was performed as previously described (Shen et al., 2017) with slight modification. After 48-h treatment of kahweol from the first-day adult stage, worms were washed thrice with M9 buffer to remove residual bacteria. Approximately 30–45 worms were transferred per well in a 96-well plate containing 50 μM H2DCF-DA solution in M9 buffer. A well with 50 μM H2DCF-DA solution in M9 buffer without worms was regarded as the background control, while a well containing 50 μM H2DCF-DA solution with paraquat was regarded as the positive control. ROS levels were recorded after 2.5 h with emission at 535 nm, excitation at 485 nm, and temperature at 25 °C using the SpectraMax i3 multimode microplate detection platform (Sunnyvale, CA, USA).

2.5. Statistical analysis

All lifespan data were analyzed with Log-rank (Mantel-Cox) tests (Table 1, Fig. 2A, Fig. 3, Fig. 4). Measurement of ROS level was analyzed with one-way ANOVA followed by Tukey's multiple comparison test (Fig. 5). Average lifespan data in Fig. 2B were analyzed with the Student t-test. All data were analyzed using GraphPad Prism ver. 9.1 (GraphPad Software, Inc., San Diego, CA, USA). Treatment was considered statistically significant when $P < 0.05$ compared to the control.

3. Result

3.1. Kahweol extended lifespan

A dose-response experiment, ranging from 10 μM to 100 μM of kahweol, was first conducted to determine the impact of kahweol on the lifespan of *C. elegans* based on previous publications (Farias-Pereira et al., 2020b. The results showed a significant lifespan extension from 10 μM of kahweol, with the maximum effect of kahweol on lifespan extension observed at 25 μM treatment, resulting in approximately 28% increase in mean lifespan compared to the control (Fig. 1 and Table 1). Kahweol, between the range of 10–50 μM, showed increased lifespan depending on concentration (Fig. 1B, $P = 0.0041$ at 10 μM, $P = 0.0010$ at 20 μM, $P < 0.0001$ at 25 μM, $P = 0.0011$ at 30 μM, and $P = 0.0005$ at 50 μM). However, greater than 30 μM of kahweol, the lifespan extension effect gradually diminished, and no significant difference in lifespan was observed at 100 μM of kahweol (Fig. 1B). Based on these, we used 25 μM of kahweol for the following experiments.

Table 1
Effect of kahweol on the median lifespan of *C. elegans*.

Strains	Kahweol (μM)	Median lifespan (days)	*P*-value	Genetic requirement
N2 (wild-type)	Control	6	<0.0001	–
	25 μM	8****		
eat-2 (ad1116)II	Control	15	0.0031	No
	25 μM	25**		
daf-2(e1370) III	Control	24	0.3363	Yes
	25 μM	22		
daf-16 (mgDf50)I	Control	13	0.2303	Yes
	25 μM	13		
tub-1 (nr2044)II	Control	12	0.9926	Yes
	25 μM	12		
sir-2.1 (ok424)IV	Control	12	0.0015	No
	25 μM	14**		
aak-2(ok524) X	Control	10	0.6219	Yes
	25 μM	10		
skn-1 (mg570)IV	Control	12	0.0001	No
	25 μM	14****		

C. elegans strains were grown and maintained at 20 °C except *daf-2 (e1370) III* was incubated at 15 °C. Kahweol was treated at 25 μM (0.1% DMSO) from day 0 (adult stage) and 0.1% DMSO to the controls. Final concentration of 120 μM FUDR was added from day 0. Media were changed and survival rate was scored every other day until all worms were dead. The median lifespan was defined as the day when the survival rate of total sample size was dropped to 50% (n = 109–129 worms/treatment). Statistical significance was analyzed with log-rank (Mantel-Cox) tests (**: $P < 0.01$, ****: $P < 0.0001$). The genetical requirement was defined by statistical significance; "Yes" at $P < 0.05$ or "No" at $P > 0.05$.

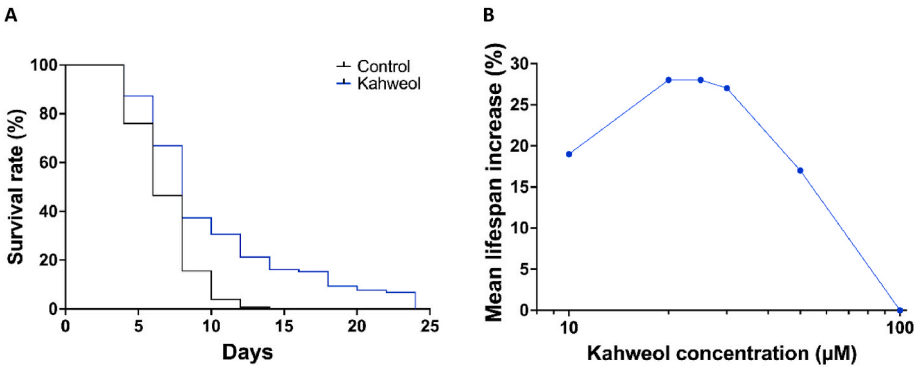

Fig. 1. Kahweol extended lifespan of wild-type *C. elegans*. Wild-type worms (N2) were treated with 25 μM kahweol (0.1% DMSO) or 0.1% DMSO as the control from the adult stage (Day 0). (A) Kahweol at 25 μM showed lifespan extension ($P < 0.0001$). (B) A dose-response was observed at concentrations of 0–100 μM of kahweol. Media were changed and the survival rate of the worms was recorded every other day until all worms were dead (n = 102–118 worms/treatment). Statistical analysis was performed using the log-rank (Mantel-Cox) test. In Fig. 1B, kahweol at 10–50 μM were significantly different from the control ($P < 0.01$).

3.2. Lifespan extension of kahweol is independent of reduced food intake

Caloric restriction is well-known in extending lifespan (Weindruch and Sohal, 1997). Based on our previous report that treating kahweol reduces food intake in *C. elegans* (Farias-Pereira et al., 2020b), we determined if kahweol's food intake reduction contributed to its effect on lifespan using *eat-2* mutant. Mutation in the *eat-2* gene causes dysfunction in the acetylcholine receptor that controls the contraction of pharyngeal muscle cells, leading to reduced food intake phenotype in *C. elegans* (Yuan et al., 2012). Treatment of kahweol at a concentration of 25 μM significantly extended the lifespan and mean lifespan of the *eat-2* null mutant ($P = 0.0209$, Fig. 2 and Table 1). This suggests that kahweol extends lifespan independent of its effect on food intake.

3.3. Kahweol extended lifespan via the insulin/insulin-like growth factor-1 signaling pathway

Next, we investigated if kahweol extends lifespan via the insulin/IGF-1 signaling (IIS) pathway, which is well known to play a crucial role in regulating the lifespan of *C. elegans* (Altintas et al., 2016; Shen et al., 2018a,b). DAF-2 is the homolog of the insulin/insulin-like growth factor-1 receptor, and it is known to be a critical factor in the IIS pathway along with DAF-16, which is the homolog of mammalian Forkhead box O transcription factor (Doerks et al., 2002; Sun et al., 2017). Activation of DAF-2 initiates a phosphorylation cascade, which retains phosphorylated DAF-16 in cellular cytoplasm, resulting in a shortened lifespan. However, when DAF-2 is inhibited, the translocation rate of DAF-16 from the cytoplasm to the nucleus increases, leading to lifespan extension (Lamitina and Strange, 2005). We examined kahweol in mutant strains *daf-2* and its primary downstream target, *daf-16* (Fig. 3A and B). Kahweol did not extend lifespans and median lifespans compared to the respective controls in both *daf-2* or *daf-16* null mutants (Fig. 3A and B and Table 1). This suggests that *daf-2* and *daf-16* are genetically required for kahweol's effects on lifespan extension.

Next, based on the observation that mutation in *tub-1* (the homolog of TUB bipartite transcription factor) is known to extend lifespan via the *daf-16*-dependent pathway in *C. elegans* (Mukhopadhyay et al., 2005), we determined if *tub-1* gene contributes to the lifespan extension effect of kahweol. Treatment of kahweol in *tub-1* null mutant failed to extend lifespan compared to the control (Fig. 3C and Table 1). These results-no significant effects of kahweol on the lifespan of *daf-2*, *daf-16*, and *tub-1* null mutant strains-indicate these genes are genetically required for kahweol's lifespan extension (Table 1).

3.4. sir-2.1 is not involved in the lifespan extension effect of kahweol

sir-2.1 is the homolog of the mammalian SIRT1 and encodes a protein belonging to the sirtuin family, which has been extensively studied for its role in aging (Zhao et al., 2020). Consistently, *sir-2.1* is another critical gene involved in lifespan regulation, which is known to be dependent on *daf-16* in *C. elegans* (Pan and Finkel, 2017; Zhao et al., 2020). Thus, kahweol's lifespan extension effect was determined by treating kahweol to *sir-2.1* null mutant strain. Our result shows that kahweol extended the lifespan of *sir-2.1* null mutant ($P = 0.0015$), indicating that *sir-2.1* is not a target of kahweol in lifespan extension (Fig. 4B and Table 1).

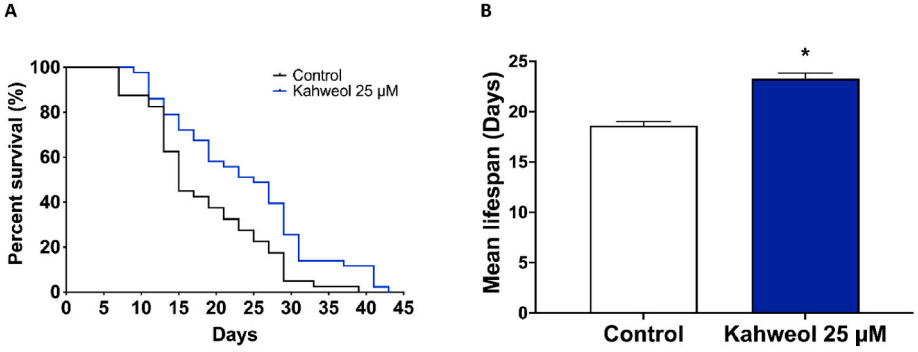

Fig. 2. Kahweol extended lifespan (A) and increased mean lifespan (B) in *eat-2* mutant. The *eat-2* null mutants were treated with 25 μM kahweol (0.1% DMSO) or 0.1% DMSO as the control from the adult stage (Day 0). Media were changed and the survival was recorded every other day until no worms survived (n = 105–125 worms/treatment). The log-rank (Mantel-Cox) test was used to analyze statistical analysis (A) and the Student t-test was used for data analysis (B). Values in B are means ± SE (n = 3, collected from 3 independent experiments). *: $P < 0.05$.

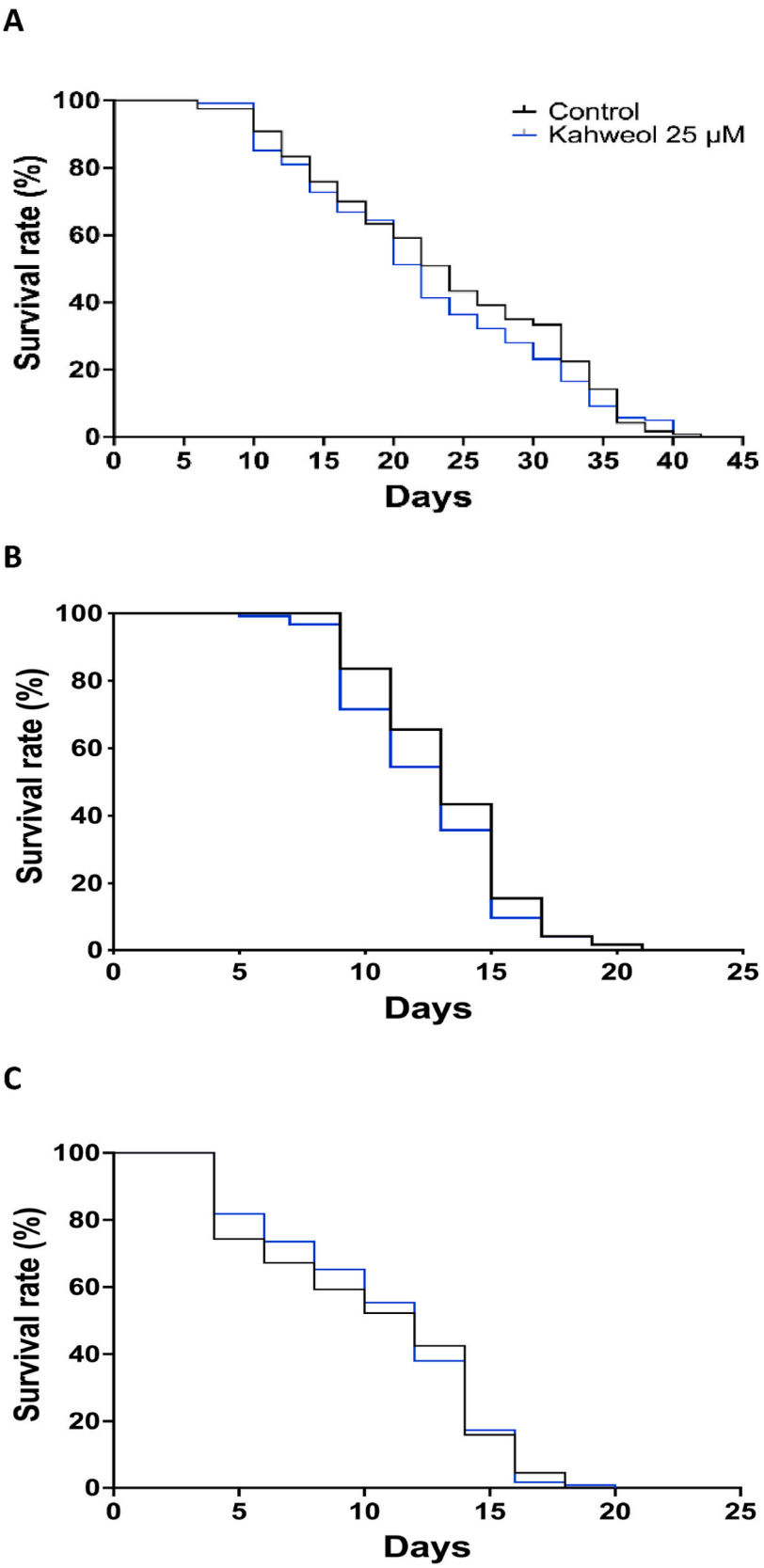

Fig. 3. Kahweol had no effects on lifespans of *daf-2* (A), *daf-16* (B), and *tub-1* (C) null mutants. *daf-2*, *daf-16*, and *tub-1* null mutants were treated with 25 μM kahweol (0.1% DMSO) or 0.1% DMSO as the control from the adult stage (Day 0). Media were changed and the survival rate was scored every other day until all worms were dead (n = 114–124 worms/treatment). For statistical analysis, the log-rank (Mantel-Cox) test was used.

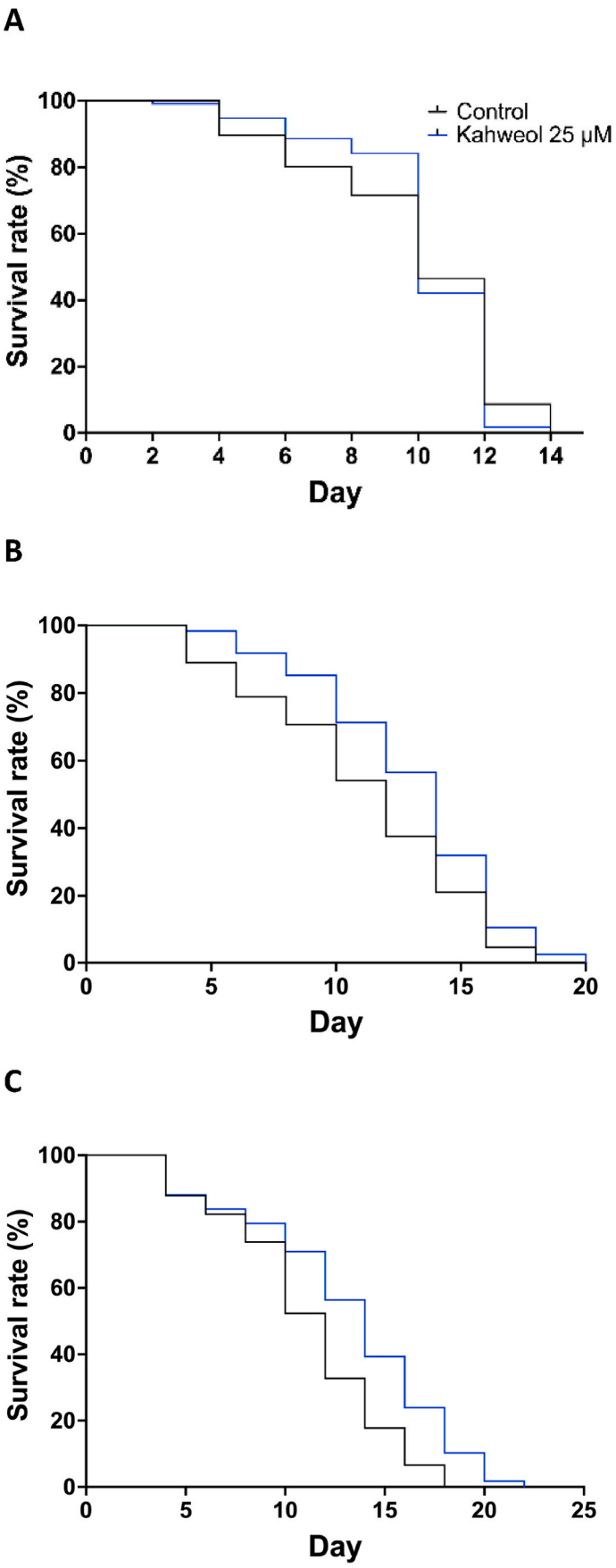

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Fig. 4. Kahweol did not extend lifespan in *aak-2* null mutant (A) but increased lifespans of *sir-2.1* (B) and *skn-1* (C) null mutants. *aak-2*, *sir-2.1*, and *skn-1* null mutants were treated with 25 μM kahweol (0.1% DMSO) or 0.1% DMSO as control at adult stage (Day 0). Media were changed and the survival rate was scored every other day until all worms were dead (n = 107–122 worms/treatment). The control group was treated with the final concentration of 0.1% DMSO, while 25 μM kahweol (0.1% DMSO) was treated. Statistical analysis was performed using the log-rank (Mantel-Cox) test. Kahweol showed significance in *sir-2.1* ($P = 0.0015$) and *skn-1* ($P < 0.0001$) compared to the respective control.

3.5. AMP-activated protein kinase (AMPK) pathway is involved in kahweols' lifespan extension

C. elegans's *aak-2* encodes a homolog of the AMPK catalytic α subunit, which regulates the lifespan by controlling energy homeostasis through regulating AMP/adenosine triphosphate (ATP) metabolism (Peng et al., 2019). Increased activity of *aak-2* leads to an extension of the lifespan, while dysfunction of *aak-2* results in a reduced lifespan in *C. elegans* (Curtis et al., 2006; Lee et al., 2008). Thus, we have used the *aak-2* null mutant strain to determine the role of the AMPK pathway in the lifespan extension effect of kahweol. Our result shows kahweol was unable to extend lifespan in *aak-2* null mutant (Fig. 4A). This indicates that *aak-2* is a genetic requirement for kahweol to extend lifespan extension in *C. elegans* (Table 1).

3.6. Kahweol does not extend lifespan via oxidative stress-related responses

Accumulation of reactive oxygen species (ROS) and oxidative stress has been reported to be closely associated with lifespan regulation (Peng et al., 2019). As kahweol has been previously reported to control the intracellular level of ROS with an *in vitro* model (Hwang and Jeong, 2008), we investigated if kahweol regulates lifespan via oxidative stress-related mechanisms. SKN-1 is a transcription factor belonging to the Nuclear respiratory factor (Nrf) family that responds to various stresses, including oxidative stress, and activation of SKN-1 pathways can change stress response and longevity (Blackwell et al., 2015). SKN-1 is also known to be regulated by the IIS pathway and AMPK pathway, which are associated with lifespan (Blackwell et al., 2015; Wang et al., 2012). Kahweol was able to extend the lifespan of the *skn-1* null mutant compared to the control ($P < 0.0001$), which indicates that kahweol does not directly target *skn-1* (Fig. 4C and Table 1). Consistently, we found that kahweol did not affect ROS levels in wild-type *C. elegans* (Fig. 5).

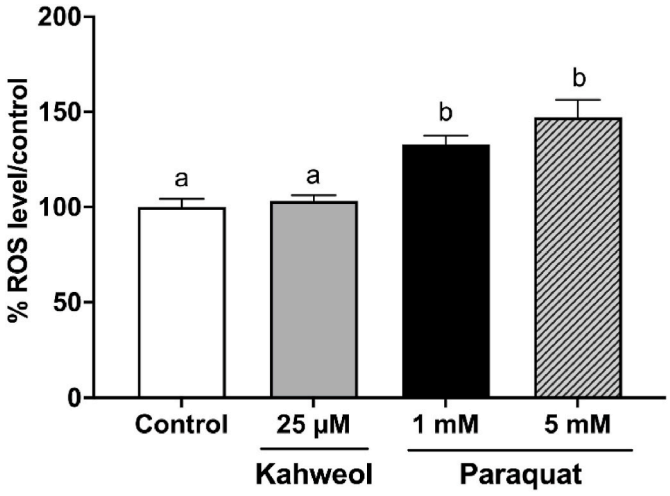

Fig. 5. Kahweol had no effects on reactive oxygen species (ROS) level of wild type *C. elegans*. Synchronized day 0 (adult stage) wild-type worms were treated with 0.1% DMSO (control) or 25 μM kahweol in 0.1% DMSO for 48 h. Paraquat at 1 mM and 5 mM were used as positive controls. Values represent means ± S. E. (n = 3, 32–44 worms per sample). Means with different letters represent significant difference at $P < 0.05$.

4. Discussion

Based on the role of caloric restriction in lifespan and our previous report of the reduced food intake by kahweol, we investigated kahweol's effect on lifespan in this study. Our result showed that kahweol significantly extended the lifespan independent of reduced food intake but found that kahweol targets multiple key genes involved in aging, including *daf-2*, *daf-16*, *tub-1*, and *aak-2*. The summary of potential mechanisms involved with kahweol on aging is summarized in Fig. 6. The role of kahweol in lifespan extension observed in the current report is the first *in vivo* study to report the potential benefits of kahweol, a diterpene found in coffee, in aging.

Caloric restriction is directly associated with the modulation of lifespan (Omodei and Fontana, 2011; Weindruch and Sohal, 1997). We first tested our hypothesis that treatment of kahweol would extend the lifespan by reducing the food intake of *C. elegans* by using the *eat-2* mutant (Yuan et al., 2012). Mutation in this gene reduces food consumption and leads to a significantly extended lifespan, as observed in Fig. 2 and Rodríguez-Palero et al. (2018). However, kahweol extended its lifespan significantly in the *eat-2* mutant strain, indicating that kahweol extend its lifespan independent of its effect on reduced food intake.

However, we observed that *daf-2* and *daf-16* genes are essential for kahweol's lifespan-extending effect. This indicates that kahweol's impact on lifespan is closely associated with regulating insulin signaling, DAF-2, and its major downstream transcription factor, DAF-16, as the IIS pathway plays a critical role in coordinating growth, metabolism, and

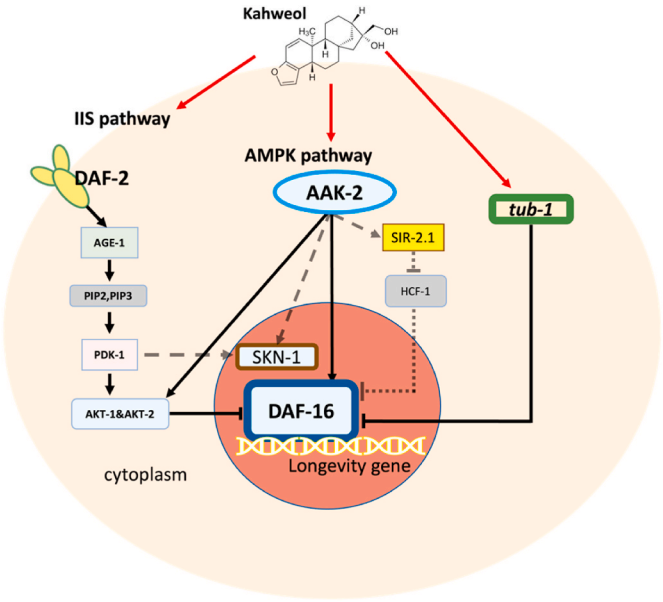

Fig. 6. Suggested mechanisms of insulin/insulin-like growth factor-1 signaling pathway (IIS) and AMP-activated protein kinase (AMPK) pathway by kahweol. DAF-16 (the homolog of Forkhead box O transcription factor) is targeted by DAF-2 (the homolog of insulin/insulin-like growth factor-1 receptor) and AAK-2 (the homolog of AMP-activated protein kinase. Within IIS pathway, signal from DAF-2 reaches DAF-16 via AGE-1 (the homolog of phosphoinositide 3 kinase), phosphatidylinositol 4,5- bisphosphate (PIP2)/phosphatidylinositol 3,4,5- trisphosphate (PIP3), PDK-1 (the homolog of mammalian Akt-PKB kinase PDK1), and AKT-1&AKT-2 (the homolog of AKT serine/threonine kinase 1 and AKT serine/threonine kinase 2) pathway. In addition, *tub-1* is suggested to regulate lifespan by modulating *daf-16*.

lifespan in *C. elegans* (Altintas et al., 2016; Lee and Lee, 2022). In *C. elegans*, activation of DAF-2 results in phosphorylation of transcription factor DAF-16, preventing it from translocating in the nucleus, leading to a shorter lifespan than wild-type (Venz et al., 2021). In contrast, inhibition of DAF-2 leads to activation of DAF-16 without phosphorylation, which then translocates DAF-16 into the nucleus (Lamitina and Strange, 2005). The activation of DAF-16, the ortholog of mammalian FOXO transcription factors, is a key downstream target of the IIS pathway that improves longevity by regulating the expression of a wide array of target genes involved in stress response, metabolism, and cellular homeostasis (Altintas et al., 2016; Lee and Lee, 2022). This suggests that the lifespan extension effect of kahweol results from the complex interplay with the IIS pathway.

TUB-1 is a protein that regulates various biological processes, including aging and fat metabolism in *C. elegans* (Elle et al., 2008; Farias-Pereira et al., 2020b). However, the lifespan-regulating effect and regulation on fat metabolism of *tub-1* have been reported to be independent. It is suggested that the *tub-1* pathway modulates the transport of essential molecules in ciliated neurons linked with the regulation of *daf-16,* which can lead to lifespan regulation. Alternatively, TUB-1 influences fat metabolism by directly controlling the transport of important molecules in ciliated neurons, mediated by the protein RBG-3 (Fan et al., 2004; Stein et al., 2003). Our previous study reported an independent role of *tub-1* on reduced fat accumulation by kahweol at 60 μM (Farias-Pereira et al., 2020b). Thus, our current finding that kahweol acts via *tub-1* may be linked to its role in *daf-16* for lifespan extension, independent of its role in fat metabolism.

While the IIS pathway mediating the lifespan extension effect of kahweol was a key finding, it is important to note that additional factors and signaling pathways may also contribute to kahweol's effect on lifespan. Although SIR-2.1 is one of the key proteins known to be associated with both the IIS pathway and AMPK pathway to regulate lifespan (Bamps et al., 2009; Tullet et al., 2008), we found that kahweol extended the lifespan of *sir-2.1* mutant strains, indicating that the sirtuin protein may not be the target of kahweol's lifespan extension effect.

Alternatively, the AMPK pathway is a critical metabolic regulator that senses cellular energy levels and coordinates various metabolic processes to maintain energy homeostasis (Garcia and Shaw, 2017). In *C. elegans*, the catalytic α subunit of AMPK is encoded by the *aak-2* (Lee et al., 2008), and activation of AMPK has been well known to extend lifespan, while dysfunction of *aak-2* leads to reduced lifespan (Apfeld et al., 2004; Uno and Nishida, 2016). Our current results indicate that kahweol targets *aak*-2 that can contribute to lifespan extension. The role of the AMPK pathway in regulating lifespan by kahweol adds further complexity to the underlying mechanisms through which kahweol impacts longevity, as AMPK is involved in regulating diverse cellular processes, such as energy metabolism, mitochondrial function, and stress response (Apfeld et al., 2004; Curtis et al., 2006; Salminen and Kaarniranta, 2012; Uno and Nishida, 2016). It is known that activation of the AMPK pathway in *C. elegans* increases the activity of *daf-16* transcription, resulting in lifespan extension (Sun et al., 2017). Also, others have reported that activation of *aak-2* phosphorylates AKT-1, which prevents DAF-16 phosphorylation, leading to the translocation of DAF-16 into the nucleus (Greer et al., 2007; Sun et al., 2017). Alternatively, it is known that SKN-1 is one of the transcription factors regulated by AMPK and involved in stress response and longevity (Blackwell et al., 2015); however, our results suggest that kahweol does not mitigate its effect on lifespan dependent on SKN-1. Thus, the precise molecular mechanism underlying how kahweol interacts with *aak-2* and its downstream effectors remains to be elucidated. Future studies are needed to investigate the specific interactions between kahweol and *aak-2*, as well as the downstream targets, such as *daf-12* (the homolog of mammalian vitamin D receptor) or *hsf-1* (the homolog of heat shock transcription factor 1), that can contribute to mediating kahweol's lifespan extension effect (Murphy and Hu, 2013; Seo et al., 2013).

ROS is a by-product of cellular metabolism; the level of ROS production increases when cellular activity increases and it is commonly known that increased ROS level can cause cellular damage and lead to reduced lifespan (Shields et al., 2021; Snezhkina et al., 2019). Thus, any treatments that can scavenge ROS can potentially extend the lifespan. Our current study showed that kahweol did not impact cellular ROS levels, and *skn-1* was not involved in its role in lifespan, indicating that kahweol does not extend lifespan via oxidative stress-mediated response. This is consistent with Cárdenas et al. (2014) reporting that kahweol has no significant effects at 10–50 μM on intracellular ROS levels in human gingival fibroblast cells. However, others reported ROS scavenging activity of kahweol after 6-hydroxydopamine treatments in human neuroblastoma, SH-SY5Y cells (Hwang and Jeong, 2008). Although our current study and Hwang and Jeong (2008) used the same method to determine ROS levels, we used an *in vivo* model of *C. elegans* with 25 μM of kahweol, while they used an *in vitro* model with a maximum concentration of 10 μM. In addition, Hwang and Jeong (2008) measured the ROS level after inducing ROS production with 6-hydroxydopamine. This suggests that kahweol may have an anti-oxidation effect depending on the experimental model, concentrations, and conditions. Thus, the significance of kahweol's role in oxidative stress responses, particularly in aging, will need to be further determined to understand its role clearly.

In the current study, we observed the dose-response of kahweol in lifespan extension, an inverted U-shape curve (Baldi and Bucherelli, 2005; Kang and Lee, 2009; Levison and Levison, 2009). As the treatment is introduced, it can initially interact with the targeted system or condition, leading to a positive response. This response continues to improve until reaching an optimal point, where the treatment has achieved its maximum efficacy (Cavassim et al., 2012; Gokduman et al., 2018; Siemiradzka et al., 2020). This is followed by diminishing or adverse effects that can result in a decline of its impact (Kar et al., 2021; Polston, 2017; Saki et al., 2013). The decrease in response after the peak can be attributed to the potential toxicity or the ability to tolerate the treatment (Gorelick, 2012; Gupta, 2016; Kar et al., 2021). Currently, no straightforward method exists to extrapolate doses used in *C. elegans* to humans. Thus, additional studies using mammals will be needed to confirm the significance of the current observations, including the proper amount of kahweol for health benefits.

Although we have found target genes and pathways of kahweol in aging, the precise mechanism still needs to be fully understood. Based on *daf-2*, *tub-1*, and *aak-2,* all can mitigate their functions on lifespan via *daf-16;* it is possible that kahweol may target *daf-16.* However, whether kahweol acts on *daf*-16 by controlling transcription, translation, post-translation, or through a combination of these processes to modulate the activity of genes and proteins within these pathways needs to be clarified. Alternatively, kahweol may mitigate its actions directly to *daf-2*, *tub-1*, and *aak-2*. Thus, future studies are required to determine whether kahweol extends lifespan primarily depending on its function on *daf-16* or directly to *daf-2*, *tub-1*, and *aak-2*.

In conclusion, our study showed that kahweol significantly extends the lifespan of *C. elegans* in a dose-dependent manner. Kahweol targets the insulin/insulin-like growth factor-1 signaling pathway and AMP-activated protein kinase pathway (Fig. 6). These target pathways, as well as target genes of *daf-2*, *daf-16*, *tub-1*, and *aak-2,* are conserved in mammals, including humans. Therefore, our findings suggest a potential health benefit of kahweol to enhance longevity and other metabolic or age-associated diseases in humans.

CRediT authorship contribution statement

Junhyo Cho: Conceptualization, Data collection, Visualization, Writing. **Yeonhwa Park:** Conceptualization, Project administration, Supervision, Funding acquisition, Writing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Given her role as the Associate Editor at the time of submission, Dr. Yeonhwa Park was not involved in the peer review of this article and had no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to another editor, as per the Journal guidelines. The authors declare that they have no other known competing financial interests that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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