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EFEITOS BIOLÓGICOS DA CURCUMINA E SUA FORMA MICRONIZADA EM ENSAIOS *IN VITRO* E *IN VIVO* EM PEIXES-ZEBRA

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Tese apresentada ao Programa de Pós-graduação em Neurociências do Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial à obtenção do título de Doutora em Neurociências.

Orientador: Prof. Dr. Angelo Luis Stapassoli Piato

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RESUMO

O estresse induz mudanças neurobiológicas complexas no sistema nervoso central que envolvem alterações nos níveis de neurotransmissores, defeitos na plasticidade sináptica e neurogênese, desequilíbrio do estado oxidativo, disfunção mitocondrial e neuroinflamação, predispondo o indivíduo a transtornos mentais como ansiedade e depressão. A farmacoterapia disponível para tratar esses transtornos apresenta limitações em relação à eficácia, justificando a busca por novos tratamentos. Nesse sentido, estudos têm demonstrado que a curcumina, um polifenol extraído do rizoma de *Curcuma longa* L. (Zingiberaceae), é um potencial candidato para tratar transtornos mentais pois é capaz de modular processos biológicos relacionados. Entretanto, a curcumina apresenta baixa biodisponibilidade e isso pode comprometer sua utilização. O processo de micronização utilizando fluido supercrítico é capaz de gerar mudanças na estrutura física, aumentar a taxa de dissolução e solubilidade de compostos ativos. Na presente tese, comparamos, através de diferentes ensaios *in vitro* e *in vivo*, os efeitos da curcumina convencional (CUR) com a curcumina micronizada (CM). Inicialmente, investigamos os efeitos antioxidantes *in vitro* da CUR e CM bem como avaliamos os efeitos comportamentais e bioquímicos das diferentes preparações de curcumina em um modelo de estresse crônico imprevisível (ECI) em peixes-zebra. Nos testes *in vitro*, CM (1 g/L) apresentou maior atividade antioxidante do que CUR, medida pelo poder antioxidante redutor de ferro (FRAP), remoção do radical 1,1-difenil-2-2-piciclo-hidrazila (DPPH) e teste de desoxirribose. O ECI aumentou a distância total percorrida no teste de interação social e diminuiu o número de cruzamentos, tempo de permanência e entradas na área superior do aquário no teste do tanque novo. Além disso, o ECI diminuiu os níveis de tióis não proteicos (NPSH), aumentou a atividade da enzima glutationa redutase (GR) e os níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS) no encéfalo de peixes-zebra. Os efeitos comportamentais induzidos pelo ECI não foram bloqueados pelas preparações de curcumina. Apesar disso, a CM apresentou propriedades antioxidantes superiores à CUR nos animais submetidos ao ECI. Posteriormente, avaliamos os efeitos comportamentais e bioquímicos de ambas as preparações de curcumina em um o modelo de estresse agudo por contenção (EAC) em peixes-zebra. O EAC aumentou o tempo de permanência na área central e o número de cruzamentos entre as áreas interna e externa do tanque e diminuiu o tempo de imobilidade dos animais no teste de tanque aberto. Além disso, o EAC induziu dano oxidativo pelo aumento dos níveis de TBARS e diminuição dos níveis de NPSH. Esses efeitos comportamentais e bioquímicos induzidos por EAC não foram bloqueados por nenhuma preparação de curcumina. Finalmente, avaliamos os efeitos comportamentais e bioquímicos de CUR e CM em larvas de peixe-zebra 7 dias pós-fertilização (dpf). A CM aumentou a distância total percorrida e o ângulo absoluto de virada no teste de tanque aberto quando comparada ao controle. Embora esses estudos não tenham medido a concentração de curcumina no encéfalo dos peixes-zebra, nossos resultados sugerem que a micronização aumenta a biodisponibilidade da curcumina, potencializando sua atividade antioxidante *in vitro* e *in vivo*. Nosso estudo agrega importantes achados que contribuem para o corpo de evidências que apoiam a utilização do processo de micronização como estratégia para aumentar a biodisponibilidade e, potencialmente, os efeitos terapêuticos de compostos ativos. Além disso, evidenciamos que a CM preveniu os efeitos de estresse crônico em marcadores neuroquímicos, apesar da ausência de um correlato comportamental evidente em peixes-zebra.

ABSTRACT

Stress induces complex neurobiological modifications in the central nervous system that involve changes in neurotransmitter levels, defects in synaptic plasticity and neurogenesis, oxidative state imbalance, mitochondrial dysfunction, and neuroinflammation, predisposing the individual to mental disorders such as anxiety and depression. The pharmacotherapy available to treat these disorders has limitations in terms of effectiveness, justifying the search for new treatments. In this sense, studies have shown that curcumin, a polyphenol extracted from the rhizome of *Curcuma longa* L. (Zingiberaceae), is a potential candidate to treat mental disorders because it can modulate biological processes related. However, curcumin has low bioavailability, and this can compromise its use. The micronization process using supercritical fluid is able of generating changes in the physical structure, increasing the dissolution rate and solubility of active compounds. In this thesis, we compared, through different *in vitro* and *in vivo* assays, the effects of conventional curcumin (CUR) with micronized curcumin (MC). Initially, we investigated the *in vitro* antioxidant effects of CUR and MC as well as evaluated the behavioral and biochemical effects of different curcumin preparations in unpredictable chronic stress (UCS) model in zebrafish. In the *in vitro* tests, MC (1 g/L) showed greater antioxidant activity than CUR, measured by the iron-reducing antioxidant power (FRAP), 1,1-diphenyl-2-2-picyclohydrazyl (DPPH) radical removal, and deoxyribose test. The UCS increased the total distance traveled in the social interaction test and decreased the number of crossings, spent time, and entries in the upper area of the tank in the novel tank test. Furthermore, UCS decreased the non-protein thiols (NPSH) levels, increased the glutathione reductase (GR) enzyme activity, and the thiobarbituric acid reactive substances (TBARS) levels in the zebrafish brain. UCS-induced behavioral effects were not blocked by any curcumin preparations. Despite this, MC showed antioxidant properties superior to CUR in animals submitted to UCS. Subsequently, we evaluated the behavioral and biochemical effects of both curcumin preparations in the acute restraint stress (ARS) model in zebrafish. The ARS increased the time spent in the central area and the number of crossings between the internal and external areas of the tank and decreased the immobility time of the animals in the open tank test. Furthermore, ARS induced oxidative damage by increasing TBARS levels and decreasing NPSH levels. These ARS-induced behavioral and biochemical effects were not blocked by any curcumin preparation. Finally, we evaluated the behavioral and biochemical effects of CUR and MC in zebrafish larvae 7 days post-fertilization (dpf). The MC increased the total distance traveled and the absolute turning angle in the open tank test when compared to control. Although these studies did not measure the concentration of curcumin in the zebrafish brain, our results suggest that micronization increases the bioavailability of curcumin, enhancing its antioxidant activity *in vitro* and *in vivo*. Our study brings together important findings that contribute to the body of evidence supporting the use of the micronization process as a strategy to increase the bioavailability and, potentially, the therapeutic effects of active compounds. Furthermore, we evidenced that MC prevented the effects of chronic stress on neurochemical markers, despite the absence of evident behavioral correlates in zebrafish.

LISTA DE TABELAS E FIGURAS

LISTA DE ABREVIATURAS

ACTH	Hormônio adrenocorticotrófico
BDNF	Fator neurotrófico derivado do cérebro
CAT	Catalase
CEUA	Comissão de ética no uso de animais
CM	Curcumina micronizada
COX-2	Ciclo-oxigenase-2
CRH	Hormônio liberador de corticotrofina
CUR	Curcumina
DPF	Dia pós-fertilização
DPPH	Radical 1,1-difenil-2-2-piciclo-hidrazila
EAC	Estresse agudo por contenção
ECI	Estresse crônico imprevisível
ERO	Espécies reativas de oxigênio
Fe^{2+}	Ferro no estado ferroso
Fe^{3+}	Ferro no estado férrico
FRAP	Poder antioxidante redutor de ferro
GPx	Glutationa peroxidase
GR	Glutationa redutase
GSH	Glutationa reduzida
GSSG	Glutationa oxidada
H_2O_2	Peróxido de hidrogênio
HHA	Hipotálamo-hipófise-adrenal
HPI	Hipotálamo-hipófise-inter-renal
IDO	Indoleamina 2,3-dioxigenase
IL-1β	Interleucina 1-β
IL-6	Interleucina 6
iNOS	Óxido nítrico sintase
MDA	Malondialdeído
$NADP^+$	Nicotinamida adenina dinucleótido fosfato oxidada
NADPH	Nicotinamida adenina dinucleótido fosfato
NF-κB	Fator nuclear-κB
NO	Óxido nítrico
NRF2	Fator nuclear relacionado ao eritroide 2
NPSH	Tiol não proteico
$O_2^{-\cdot}$	Ânion superóxido
$OH\cdot$	Radical hidroxila
SEDS	Dispersão de solução aumentada por fluidos supercríticos
SNA	Sistema nervoso autônomo
SNC	Sistema nervoso central
SOD	Superóxido dismutase
TBARS	Substâncias reativas ao ácido tiobarbitúrico
TNF-α	Fator de necrose tumoral α

SUMÁRIO

1. INTRODUÇÃO

1.1 Estresse

Estresse se refere ao conjunto de respostas fisiológicas e comportamentais desencadeadas pelo organismo frente a uma ameaça. O conceito de alostase, refere-se a esse processo ativo e adaptativo onde o organismo responde aos diferentes eventos estressores. Na alostase ocorre a ativação do sistema nervoso autônomo (SNA) simpático, resultando na liberação de catecolaminas, e do eixo hipotálamo-hipófise-adrenal (HHA), resultando na liberação de glicocorticoides. Apesar da alostase ser um processo adaptativo e fundamental em resposta às demandas do dia a dia, a ativação dos eixos neuroendócrinos de maneira sustentada pode causar efeitos deletérios ao organismo e isso é chamado de sobrecarga alostática. A sobrecarga alostática está associada a etiologia de doenças periféricas e centrais (MCEWEN, 2006; MCEWEN et al., 2015a).

As respostas que ocorrem devido ao estresse são mediadas por circuitos amplamente sobrepostos no prosencéfalo límbico, hipotálamo e tronco encefálico, de modo que as respectivas contribuições dos sistemas neuroendócrino (glicocorticoides) e autonômico são sintonizados de acordo com a modalidade e intensidade do estressor. As regiões límbicas responsáveis por regular as respostas ao estresse se cruzam com os circuitos responsáveis pela memória e recompensa, fornecendo um meio de adaptar a resposta ao estresse em relação a experiências anteriores e aos resultados esperados. O SNC desencadeia respostas de estresse proporcionais à natureza do estímulo. Estressores físicos (perda de sangue, infecção e dor) requerem uma reação imediata que é desencadeada por mecanismos reflexivos. Já estressores não físicos (psicogênico) com base em experiências anteriores ou programações inatas, requerem processamento em múltiplas estruturas límbicas do prosencéfalo, incluindo a amígdala, o hipocampo e o córtex pré-frontal. Essas regiões recebem informações associativas de áreas subcorticais e corticais que estão envolvidas no processamento sensorial de ordem superior (núcleos olfatórios, córtex piriforme e córtex insular) e memória (septo medial, córtex entorrinal

e córtex cingulado). Eles também recebem aferências de áreas envolvidas em atenção e excitação (*locus ceruleus* e núcleos da rafe). As eferências dessas estruturas límbicas converge em locais subcorticais, permitindo o processamento das informações límbicas e atuando em paralelo para influenciar a ativação do eixo HHA e respostas autonômicas (HERMAN et al., 2003; ULRICH-LAI; HERMAN, 2009).

O SNA é responsável pelo controle das funções vegetativas, como respiração, batimentos cardíacos, circulação do sangue, controle de temperatura e digestão. Durante a ativação frente ao estresse, o sistema simpático predomina, mediando alterações importantes no organismo desencadeadas por descargas adrenérgicas da medula da suprarrenal e de noradrenalina em neurônios pós-ganglionares, como demonstradas na tabela 1 (KVETNANSKÝ et al., 1995; PACAK; MCCARTY, 2007).

Tabela 1 – Alterações fisiológicas desencadeadas pela ativação do SNA simpático durante a resposta de estresse.

Alterações	Objetivos
Aumento da frequência cardíaca e pressão arterial	Permitir que o sangue circule mais rapidamente, facilitando a chegada de oxigênio e nutrientes aos músculos esqueléticos e encéfalo para mobilidade e o movimento
Contração do baço	Levar mais hemácias à corrente sanguínea, facilitando a oxigenação
Glicogenólise no fígado	Prover energia para os músculos e encéfalo
Redistribuição sanguínea	Diminuir o fluxo para a pele e vísceras e aumentar para músculos e encéfalo
Aumento da frequência respiratória e dilatação dos brônquios	Favorecer a captação de oxigênio
Dilatação pupilar e exoftalmia	Aumentar a eficiência visual
Aumento do número de linfócitos na corrente sanguínea	Reparar possíveis danos aos tecidos eventualmente causados por agentes estressores

Fonte: Adaptado de Pacak; mccarty (2007).

O eixo HHA é responsável por desencadear respostas mais lentas e prolongadas, desempenhando um papel crucial na adaptação do organismo ao estresse. Conforme

demonstrado na figura 1, o eixo HHA inicia em uma região específica do SNC denominada hipotálamo, onde neurônios do núcleo paraventricular secretam o hormônio liberador de corticotrofina (CRH). O CRH por sua vez, estimula a glândula hipófise, localizada logo abaixo do hipotálamo, a sintetizar e liberar o hormônio adrenocorticotrófico (ACTH), além de outros neuro-hormônios e peptídeos cerebrais, como beta-endorfinas (que modificam o limiar para dor) somatotrofina (acelera o metabolismo) e prolactina (participação no sistema imune). Por fim, o ACTH atinge a corrente sanguínea e atua estimulando a síntese e liberação do cortisol, um hormônio esteroide secretado pela glândula suprarrenal (também chamada de glândula adrenal). A ativação da amigdala leva a liberação de CRH pelo hipotálamo, de forma a estimular o eixo, enquanto que, a interação dos glicocorticoides com os receptores de glicocorticoides localizados no córtex pré-frontal, no hipocampo, nos neurônios parvocelulares do núcleo paraventricular do hipotálamo e na hipófise ou com os receptores de mineralocorticoides localizados no hipocampo levam a uma inibição da liberação de cortisol, estabelece-se, dessa forma, um mecanismo de retroalimentação negativa, com o cortisol atuando de forma a inibir o eixo HHA. (CERNACKOVA et al., 2020; HILLER-STURMHÖFEL; BARTKE, 1998; MCEWEN et al., 2015a).

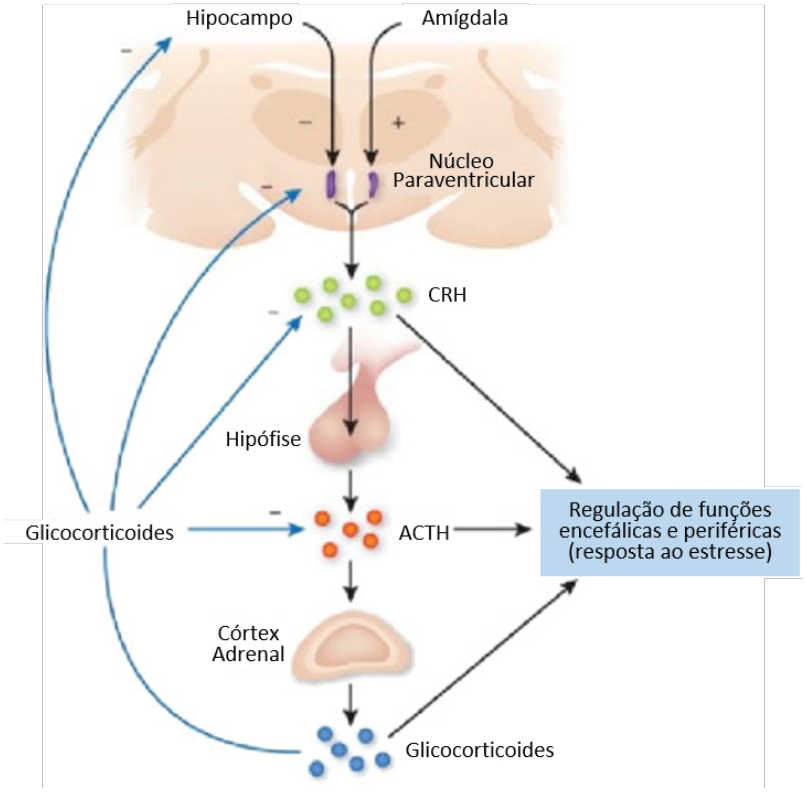

Figura 1. Representação esquemática do eixo HHA. HHA (hipotálamo-hipófise-adrenal), CRH (hormônio liberador de corticotrofina), ACTH (hormônio adrenocorticotrófico), + (ativação), - (inibição). Fonte: Adaptado de Liyanarachchi; Ross; Debono (2017).

No estresse crônico, a carga alostática é mantida superando as capacidades adaptativas do organismo, ocorrendo uma sobrecarga alostática que pode ocasionar efeitos deletérios. Neste caso, hiperativação do sistema nervoso autônomo simpático e eixo HHA, aumento dos níveis de cortisol, disfunção dos sistemas neurotransmissores, neuroinflamação, excitotoxicidade glutamatérgica, defeitos na neurogênese e plasticidade sináptica, disfunção mitocondrial, desequilíbrio do estado redox e dano oxidativo podem estar presentes (CERNACKOVA et al., 2020; LUPIEN et al., 2009; MCEWEN et al., 2015a; MILLER; RAISON, 2016; SAPOLSKY; KREY; MCEWEN, 1986; ULRICH-LAI; HERMAN, 2009). No sistema periférico, a hiperatividade do eixo HHA, o aumento da liberação de catecolaminas e a inflamação podem convergir em doenças cardiovasculares e metabólicas crônicas como obesidade e diabetes mellitus

(OTTE et al., 2016). Em última instância, a sobrecarga alostática resultante do estresse crônico causa atrofia de neurônios no hipocampo e córtex pré-frontal, regiões do encéfalo envolvidas na memória, atenção seletiva e função executiva, e causa hipertrofia dos neurônios na amígdala, envolvida no medo e na ansiedade, bem como a agressão. Assim, essas modificações estruturais podem levar a alterações comportamentais principalmente relacionadas à motivação, sono, cognição, capacidade de tomada de decisões, sexo, bem como, aumento da evitação, alarme (ansiedade) e agressividade, que caracterizam diversos transtornos neuropsiquiátricos, incluindo ansiedade e depressão (CHATTARJI et al., 2015; MCEWEN, 2006; MURRAY; FELLOWS, 2022).

1.2 Transtornos Mentais

Os transtornos mentais são condições altamente incapacitantes com fisiopatologia multifatorial complexa que envolve interações de fatores genéticos e ambientais. Essas condições resultam em mudanças significativas no pensamento, emoção e/ou comportamento e estão associadas a angústia e/ou problemas de funcionamento nas atividades sociais, profissionais ou familiares (PAREKH, 2018).

Transtornos mentais incluindo ansiedade e depressão são as principais causas de anos de vida perdidos por incapacidade, calculados por uma estimativa global da população entre os anos de vida perdidos para a mortalidade prematura e os anos vividos com incapacidades, representando 37% dos anos de vida perdidos por doenças não transmissíveis (INSEL et al., 2013; WHITEFORD et al., 2015). Em 2010, os transtornos mentais tiveram um custo para a economia global de US$ 2,5 trilhões e a estimativa é aumentar US$ 6,5 trilhões até 2030, sendo condições crônicas que mais gastam em todo o mundo (INSEL et al., 2013).

Embora tratamentos para esses transtornos estejam disponíveis, há um número significativo de pacientes que não respondem adequadamente à terapia, contribuindo ainda mais para a carga global desses transtornos mentais (MILLER; RAISON, 2016; RAMAHOLIMIHASO; BOUAZZAOUI; KALADJIAN, 2020). Os transtornos de ansiedade são os mais comuns dos transtornos mentais e afetam quase 30% dos adultos

em algum momento de suas vidas (APA, 2021). Seguida pela depressão que afeta cerca de 6% da população adulta por ano (OTTE et al., 2016; WHO, 2021). Aproximadamente 280 milhões de pessoas no mundo têm depressão, além disso, mesmo com o tratamento, possuí uma taxa de recorrência de pelo menos 45% entre esses pacientes, sendo a desistência do uso de medicação a principal causa (OTTE et al., 2016; WHO, 2021).

Para o tratamento de transtornos de ansiedade e de humor são utilizados antidepressivos, incluindo os antidepressivos inibidores seletivos da recaptação da serotonina (por exemplo, fluoxetina) e os inibidores da recaptação da serotonina e noradrenalina (por exemplo, venlafaxina), entre outros (OTTE et al., 2016; WHO, 2021). Na depressão, cerca de 50-60% dos pacientes não obtém respostas adequadas após um primeiro tratamento com antidepressivos (OTTE et al., 2016). Do ponto de vista clínico, a não adesão aos antidepressivos continua elevada por vários motivos, sendo os efeitos adversos (por exemplo náusea, insônia, dores de cabeça, tonturas, sintomas gastrointestinais, disfunção sexual, ganho de peso e distúrbios de sono) uma das principais causas e um fator limitante para a manutenção em longo prazo (OTTE et al., 2016; WHO, 2021). Além disso, os antidepressivos apresentam uma latência para o aparecimento dos efeitos terapêuticos que normalmente leva entre 2 a 8 semanas, contribuindo muitas vezes para a descontinuação do tratamento (OTTE et al., 2016; WHO, 2021). Portanto, justifica-se a busca por novos compostos com mecanismo de ação capaz de modular processos biológicos distintos e que apresentem menor incidência de efeitos adversos.

1.3 Estresse oxidativo

A base neurobiológica dos transtornos mentais não é totalmente compreendida, mas estudos mostraram que desequilíbrios do estado oxidativo e maior produção de espécies reativas de oxigênio (ERO), são achados comuns em pacientes com transtornos de humor e/ou ansiedade (FEDOCE et al., 2018). O aumento na geração de radicais livres a partir ERO, como superóxido ($O_2^{\cdot -}$), peróxido de hidrogênio (H_2O_2) e radical

hidroxila (OH·), associada a uma diminuição das defesas antioxidantes causam um estado de estresse oxidativo que altera a homeostase neuronal e favorece a ocorrência de lesões oxidativas em proteínas, lipídios e ácidos nucléicos, resultando em morte celular (AVERY, 2011; FEDOCE et al., 2018; HALLIWELL, 2007; MORRIS et al., 2020; UTTARA et al., 2009; VALKO et al., 2007).

Em homeostase, a produção de ERO é controlada por diversos sistemas antioxidantes enzimáticos e não enzimáticos, como demonstrado na figura 2. A enzima superóxido dismutase (SOD) catalisa a dismutação do $O_2^{\cdot -}$ a H_2O_2, utilizando ferro ou manganês como cofator e então, o H_2O_2 é eliminado pela enzima catalase (CAT) no citosol ou pela glutationa peroxidase (GPx) na mitocôndria. A glutationa (GSH) é sintetizada a partir do dipeptídeo γ em combinação com a glicina pela ação da glutationa sintetase, consistindo em três aminoácidos, glutamato, cisteína e glicina. A GSH reduzida é um dos principais componentes antioxidantes envolvidos na remoção de ROS e manutenção do estado oxidativo. GPx reduz H_2O_2 através da oxidação de GSH a glutationa oxidada na forma dimerizada (GSSG). GSSG é então reciclado pela enzima glutationa redutase (GR) através da reação de oxidação NADPH (nicotinamida adenina dinucleótido fosfato) em NADP oxidado. Além disso, o GSH também pode sofrer oxidação e formar dissulfetos do tipo GSSR com o tiol de cisteína presente nas proteínas. O grupo sulfidrila presente no tiol de cisteína é o sítio ativo e é responsável por suas funções protetoras contra o estresse oxidativo. Portanto, sua oxidação leva à formação de dissulfetos de GSH e inativação de sua capacidade antioxidante, deixando o organismo mais suscetível a sofrer danos oxidativos (AVERY, 2011; FEDOCE et al., 2018; HALLIWELL, 2007; MAES et al., 2011; MANDELKER, 2008; MORRIS et al., 2020; UTTARA et al., 2009; VALKO et al., 2007).

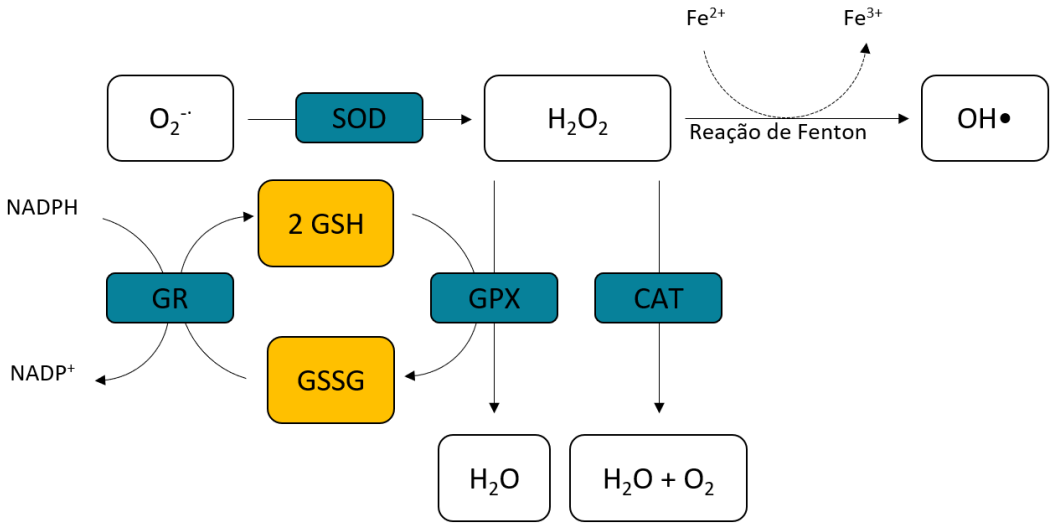

Figura 2. Enzimas envolvidas na geração e inativação de espécies reativas de oxigênio. O $O_2^{-\cdot}$ é convertido em H_2O_2 pela SOD. O H_2O_2 pode sofrer conversão espontânea para OH· por meio da reação de Fenton. O OH· é extremamente reativo e ataca a maioria dos componentes celulares. O H_2O_2 pode ser desintoxicado via GPx ou CAT para H_2O e O_2. $O_2^{-\cdot}$ (ânion superóxido), OH· (radical hidroxila), H_2O_2 (peróxido de hidrogênio), SOD (superóxido dismutase), GPx (glutationa peroxidase), CAT (catalase), O_2 (Oxigênio), Fe^{2+} (Ferro no estado ferroso), Fe^{3+} (Ferro no estado férrico), NADPH (nicotinamida adenina dinucleótido fosfato), $NADP^+$ (nicotinamida adenina dinucleótido fosfato oxidada). Fonte: Adaptado de Mandelker (2008).

Alguns órgãos, como o encéfalo, são mais vulneráveis aos efeitos prejudiciais das ERO, pois apresentam uma alta taxa metabólica e níveis mais baixos de antioxidantes (MAES et al., 2011; MANDELKER, 2008; MORRIS et al., 2020). A adaptação ao estresse (alostase) aumenta a demanda de energia cerebral, refletindo o aumento da atividade mitocondrial dentro do encéfalo, que está relacionado ao alto consumo de oxigênio e maior produção de ERO (AVERY, 2011; HALLIWELL, 2007; HALLIWELL; GUTTERIDGE, 1985; MORRIS et al., 2020; PICARD et al., 2018). O aumento da produção de ERO tem sido associado à hiperativação do eixo HHA com um consequente aumento na secreção de cortisol, por produzir danos aos neurônios do hipocampo, que regulam o feedback negativo do eixo. O cortisol também pode modular a homeostase do cálcio mitocondrial por meio da liberação de glutamato e a gerar ERO.

Além disso, o cortisol pode levar a ativação anormal da micróglia e produzir altos níveis de citocinas inflamatórias, como fator de necrose tumoral α (TNF-α), Interleucina 1β (IL-1β) e fator nuclear κB (NF-κB), como consequência ocorre aumento da geração de espécies reativas como $O_2^{-\cdot}$, cuja principal fonte na micróglia é a NADPH oxidase. O fator nuclear relacionado ao eritroide 2 (Nrf2) é responsável pela homeostase redox, resistência neuronal a estresse oxidativo e excitotoxicidade induzida por glutamato e na modulação da ativação de macrófagos em resposta à neuroinflamação. A ativação de Nrf2 por agentes eletrolíticos protege as células, principalmente pela ativação de genes relacionados a enzimas antioxidantes como a glutationa peroxidase. A liberação de NF-κB, bem como, uma super produção de ERO, estão relacionadas a uma supressão de Nrf2, contribuindo assim para um estado de estresse oxidativo envolvido em transtornos mentais e neurodegenerativos (FEDOCE et al., 2018; MORRIS et al., 2020).

1.4 Peixes-zebra como organismo modelo

A utilização do peixe-zebra (*Danio rerio*, F. Hamilton 1822) na neurociência cresceu acentuadamente nas últimas décadas, por ser uma espécie de vertebrados com características fisiológicas e genéticas homólogas aos seres humanos (CACHAT et al., 2010; MACRAE; PETERSON, 2015). Estudos demonstraram que 71% dos genes que codificam proteínas no genoma humano são relacionados a genes encontrados no genoma do peixe-zebra e 84% dos genes associados a doenças humanas possuem um ortólogo em peixes-zebra (HOWE et al., 2013).

A translação das condições humanas nos peixes-zebra permite a descoberta de potenciais alvos terapêuticos e suas interações moleculares subjacentes (KHAN et al., 2017). A arquitetura geral, características neuroanatômicas e a morfologia celular do SNC dos peixes-zebra são geralmente semelhantes à dos mamíferos (MACRAE; PETERSON, 2015). Além disso, apresenta neuroquímica conservada, compartilhando os mesmos principais neurotransmissores, receptores e transportadores dos mamíferos (STEWART et al., 2015). Por exemplo, o sistema serotonérgico se desenvolve entre 1–5 dias pós-fertilização (dpf), o sistema adrenérgico começa a aparecer 24 horas após a

fertilização (hpf) e completa-se com 5 dpf, os neurônios dopaminérgicos são detectados em 18-19 hpf no diencéfalo ventral que ascende ao estriado e se assemelha ao sistema nigroestriatal mamífero, enquanto que a distribuição de neurônios gabaérgicos em 3 dpf, já é homologa à mamíferos. Assim, o peixe-zebra é sensível à maioria das classes farmacológicas utilizadas na clínica, como os ansiolíticos e os antidepressivos (BASNET et al., 2019; KHAN et al., 2017; PANULA et al., 2010; STEWART et al., 2015).

O peixe-zebra apresentam muitas vantagens para o estudo de doenças humanas, demonstrando um amplo repertório comportamental, variando de agressão e ansiedade, a memória de longo e curto prazo, discriminação de objeto e preferência de cor e interação social, os quais podem ser modulados por diferentes intervenções farmacológicas ou físicas (BENVENUTTI et al., 2021c; CHAMPAGNE et al., 2010; FONTANA et al., 2021; GUTIÉRREZ et al., 2020; PIATO et al., 2011a). Além disso, entre as vantagens também incluem os baixos custos de manutenção, um curto ciclo de reprodução e desenvolvimento rápido e sincronizado. Os embriões são grandes e transparente e as larvas são adequadas para estudos em grande escala (BASNET et al., 2019; STEWART et al., 2015). As larvas de peixes-zebra também exibem numerosas respostas comportamentais complexas e podem ser expostas a drogas e outras pequenas moléculas simplesmente imergindo-as em solução. Além disso, os peixes-zebra no estágio larval podem ocupar diferentes nichos ecológicos e enfrentam diferentes predadores em comparação com aqueles dos peixes-zebra adulto e, portanto, o comportamento e as preferências, podem não ser necessariamente comparáveis ao dos peixes-zebra adulto (COLWILL; CRETON, 2011; SPENCE et al., 2008). Essas características tornam o peixe-zebra um excelente organismo modelo para estudo sobre as bases neurobiológicas dos transtornos mentais bem como para a identificação de novas compostos para o tratamento dessas condições (BASNET et al., 2019; KHAN et al., 2017).

1.5 Modelos de estresse em peixe-zebra

As respostas ao estresse no peixe-zebra também são mediadas pelas catecolaminas e pelo eixo hipotálamo-hipófise-inter-renal (HHI), que é funcional e estruturalmente homólogo ao eixo HHA nos mamíferos. O eixo HHI inclui o hipotálamo (especialmente núcleo pré-óptico, homólogo ao núcleo paraventricular) que, após exposição ao estresse, induz a cascata de liberação de CRH e ACTH para estimular a síntese e liberação de cortisol pelo tecido inter-renal (ALSOP; VIJAYAN, 2009). As catecolaminas são liberadas quase imediatamente após a exposição ao estresse, e os glicocorticoides são ativados pelo CRH posteriormente (DEMIN et al., 2021a; GHISLENI et al., 2012). O cortisol é um importante hormônio glicocorticoide que regula múltiplas funções fisiológicas envolvidas nas respostas ao estresse no peixe-zebra assim como em humanos (GHISLENI et al., 2012).

O modelo de estresse crônico imprevisível (ECI) em peixe-zebra foi desenvolvido para mimetizar algumas modificações comportamentais e fisiológicas desencadeadas tanto em roedores quanto em humanos (PIATO et al., 2011a). Esse modelo consiste em submeter os animais a situações imprevisíveis de exposição ao estresse de diferentes naturezas (físicos, químicos e sociais) por períodos prolongados. ECI induz alterações comportamentais, bioquímicas e moleculares semelhantes às observadas em pacientes com depressão e/ou ansiedade (MARCON et al., 2016; PIATO et al., 2011a). Nesse modelo, são observadas alterações de comportamento exploratório, aversivo, social e agressividade, além de déficits cognitivos, aumento da expressão de genes de biomarcadores pró-inflamatórios como ciclooxigenase 2 (COX-2) e interleucina 6 (IL-6), diminuição dos níveis de monoaminas, aumento de cortisol, espécies reativas de oxigênio e dano oxidativo (CHAKRAVARTY et al., 2013; MARCON et al., 2018; PIATO et al., 2011a; SHAMS; CHATTERJEE; GERLAI, 2015; SONG et al., 2018). Antidepressivos, ansiolíticos e/ou antioxidantes mostraram efeitos protetores neste modelo (DEMIN et al., 2021a; MARCON et al., 2016, 2019; MOCELIN et al., 2019a; SONG et al., 2018).

No peixe-zebra, o estresse agudo pode ser desencadeado por vários estímulos, como novidade, feromônio de alarme ou exposição a predador, perseguição por rede, contenção, aglomeração, isolamento social, hipóxia, alteração dos parâmetros da água (pH, salinidade e temperatura) ou exposição à luz brilhante (ABREU et al., 2014; BERTELLI et al., 2021; CHAMPAGNE et al., 2010; DAL SANTO et al., 2014; EGAN et al., 2009, 2009; FONTANA et al., 2021; GHISLENI et al., 2012; GIACOMINI et al., 2016; IDALENCIO et al., 2015; MOCELIN et al., 2015; PANCOTTO et al., 2018; PIATO et al., 2011b; REIS et al., 2020). O modelo de estresse agudo por contenção (EAC) consiste em restringir a habilidade do animal de se mover e escapar de um pequeno ambiente a um ponto que causa excitação psicológica e física (estresse) (CHAMPAGNE et al., 2010). Em resposta a um estressor agudo como contenção, o peixe-zebra exibe um repertório comportamental e fisiológico complexo, incluindo ansiedade, distúrbio locomotor (distância aumentada, velocidade média, movimentos erráticos, congelamento), aumento no comportamento de tigmotaxia (comportamento de nadar rente a paredes de um aquário), comprometimento cognitivo, aumento do cortisol e desequilíbrio do estado oxidativo (ABREU et al., 2014; BERTELLI et al., 2021; CHAMPAGNE et al., 2010; DAL SANTO et al., 2014; EGAN et al., 2009; FONTANA et al., 2021; GHISLENI et al., 2012; GIACOMINI et al., 2016; IDALENCIO et al., 2015; MOCELIN et al., 2015; PANCOTTO et al., 2018; PIATO et al., 2011a; REIS et al., 2020). Esses efeitos também são bloqueados por ansiolíticos, antidepressivos e/ou antioxidantes (ABREU et al., 2014; EGAN et al., 2009; GIACOMINI et al., 2016; MOCELIN et al., 2015; PANCOTTO et al., 2018; REIS et al., 2020).

Dessa forma, os protocolos de estresse crônico e agudo em peixes-zebra podem servir como uma ferramenta de *screening* para avaliação de novas drogas para o tratar transtornos psiquiátricos com etiologias relacionadas ao estresse.

1.6 Curcumina

A curcumina é um dos compostos extraídos das raízes do açafrão da terra (*Curcuma longa* L. Zingiberaceae) (figura 3). É um fenol natural amplamente utilizado em países asiáticos como corante e aditivo alimentar. Esse composto possuiu atividade antioxidante, anti-inflamatória, neuroprotetora, imunomoduladora e antidepressiva demonstrada em diversos modelos animais e em humanos (RADHAKRISHNA PILLAI et al., 2004; REETA; MEHLA; GUPTA, 2010; YADAV et al., 2005; ZHANG; LI; ZHANG, 2020).

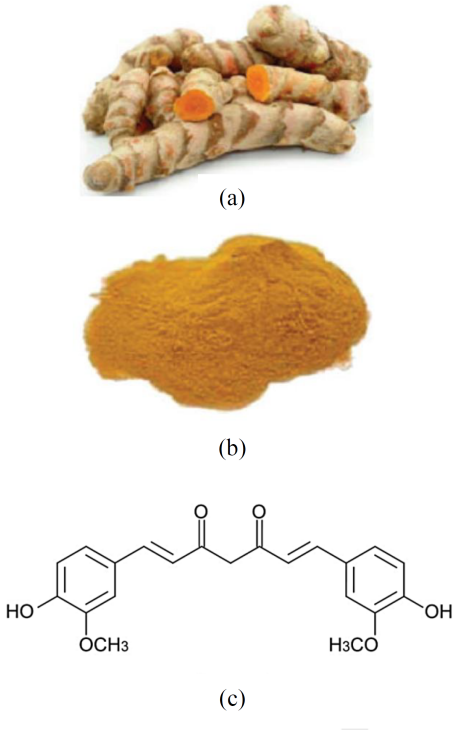

Figura 3. Raízes de *Curcuma longa* L. (Zingiberaceae) (açafrão, Fig. 3a), curcumina em pó (Fig. 3b) e estrutura química da curcumina (Fig. 3c). Fonte: Zhang et al. (2013).

Os efeitos da curcumina têm sido observados em diversos modelos de depressão em animais (Figura 4) (DA SILVA MARQUES et al., 2021; KULKARNI; BHUTANI; BISHNOI, 2008; SANMUKHANI; ANOVADIYA; TRIPATHI, 2011; WANG et al.,

2008, 2014; ZHANG; LI; ZHANG, 2020; ZHAO et al., 2014). Em modelo de estresse crônico em roedores, a curcumina diminuiu os níveis de corticosterona, aumentou a atividade de enzimas mitocondriais (complexo I a V) e antioxidantes CAT, SOD e GPx, níveis de GSH e diminuiu níveis de MDA e nitrito. Além disso, aumentou os níveis de BDNF e monoaminas e diminuiu a expressão de citocinas pró-inflamatórias como NF-κβ, IL-1β, IL-6, TNF-α, COX-2, além de reduzir o tempo de imobilidade nos testes de nado forçado e de suspensão pela cauda e aumentar a preferência por sacarose (BHUTANI; BISHNOI; KULKARNI, 2009; DA SILVA MARQUES et al., 2021; KULKARNI; BHUTANI; BISHNOI, 2008; LOPRESTI et al., 2014; PANAHI et al., 2015; WANG et al., 2008, 2014; XU et al., 2005; ZHANG; LI; ZHANG, 2020).

A curcumina também tem sido estudada como tratamento de doenças neurodegenerativas devido ao seu potencial anti-inflamatório e antioxidante, capaz de retardar a progressão da doença de Alzheimer, reduzindo a formação de placas β-amiloide no SNC (YANG et al., 2005) e a produção de homocisteína que é um fator de risco para doença vascular e atrofia cerebral na doença de Parkinson (MANSOURI et al., 2012). Em peixes-zebra, curcumina já demonstrou efeitos antioxidante contra radicais livres (ARTEAGA et al., 2021; ENDO et al., 2020; KIM et al., 2021), foi capaz de prevenir crises epiléptica aguda em animais expostos ao pentilenotetrazol (BERTONCELLO et al., 2018; CHOO et al., 2021), impediu o comprometimento cognitivo induzido pelo extrato da fumaça de cigarro nos testes de reconhecimento de cor, preferência de área, preferência horizontal e labirinto em T, além disso, atenuou as alterações bioquímicas como atividade de acetilcolinesterase e níveis de TBARS e GSH no encéfalo (MUTHURAMAN et al., 2019), bem com, preveniu comprometimento cognitivo induzidos pela escopolamina no teste de esquiva inibitória (CORADINI et al., 2021).

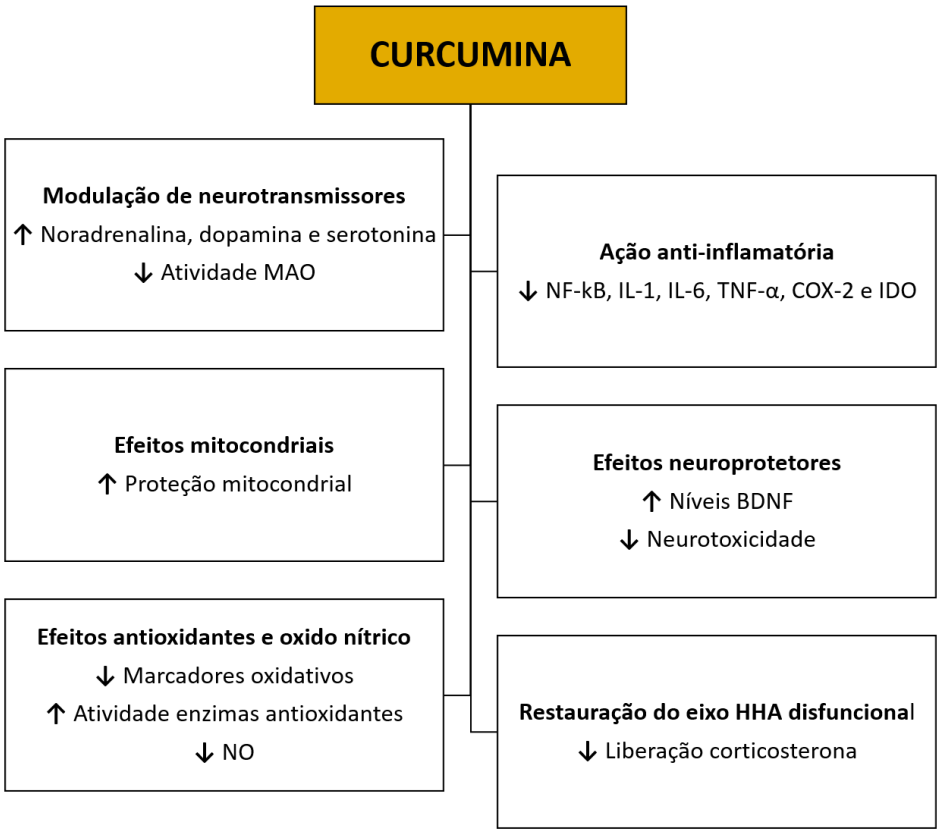

Figura 4. Potenciais mecanismos de curcumina para uso em transtornos mentais. NF-κB (fator nuclear-κB), IL-1β (interleucina 1-β), IL-6 (interleucina 6), TNF-α (fator de necrose tumoral), COX-2 (ciclo-oxigenase-2), IDO (indoleamina 2,3-dioxigenase), BDNF (fator neurotrófico derivado do cérebro), NO (óxido nítrico). Fonte: modificado de Zhang et al. (2020).

O estresse oxidativo é um indicador importante no progresso de transtornos mentais e neurodegenerativos e antioxidantes parecem apresentar efeitos protetores em modelos animais e humanos (AVERY, 2011; FEDOCE et al., 2018; HALLIWELL, 2007; MORRIS et al., 2020; PICARD et al., 2018; VALKO et al., 2007). Vários estudos demonstraram de forma consistente que o CUR tem uma potente atividade antioxidante *in vitro* e *in vivo* (AK; GÜLÇIN, 2008; MENON; SUDHEER, 2007; ZHENG et al., 2017). CUR é capaz de atenuar a produção intracelular de ERO, aumentar a atividade de enzimas antioxidantes e proteger as mitocôndrias do estresse oxidativo em ratos. Além disso, a curcumina pode não apenas mediar a neuroproteção contra disfunção mitocondrial modulando os complexos I e II pela ativação do Nrf2, mas também

suprimindo a produção de ERO e o aumentando a expressão dos genes relacionados com atividades antioxidantes enzimáticas (AK; GÜLÇIN, 2008; MENON; SUDHEER, 2007; WANG et al., 2014; ZHANG; LI; ZHANG, 2020; ZHAO et al., 2014).

No entanto, apesar desses efeitos já demonstrados na literatura, a curcumina apresenta baixa biodisponibilidade e absorção, rápido metabolismo e eliminação sistêmica, sendo degradada cerca de 80% no trato gastrointestinal, o que pode comprometer sua utilização para as diversas condições que afetam o SNC (ANAND et al., 2007a; YANG et al., 2007). Uma alternativa para melhorar a biodisponibilidade e potencializar os seus efeitos consiste no processo de micronização da matéria-prima através da técnica de fluído supercrítico (AGUIAR et al., 2017).

1.7 Micronização

O processo de micronização é caracterizado pela redução do tamanho médio das partículas a fim de proporcionar mudanças na estrutura física. Este processo é aplicado em diferentes materiais com diversos objetivos nas indústrias química, farmacêutica e alimentícia. Existem muitas vantagens na redução do tamanho de partícula de compostos ativos, como modificar o tamanho de partícula, porosidade e densidade o que permite a incorporação de um ingrediente ativo de modo que a administração possa ser direcionada para um alvo específico (AGUIAR et al., 2016). A técnica de dispersão de solução aumentada por fluidos supercríticos (Solution Enhanced Dispersion by Supercritical Fluids, na sigla em inglês SEDS) demonstrada na figura 5, é um método que pode ser aplicado para micronizar compostos com propriedades bioativas. Já foi comprovado que a micronização por essa técnica não gera degradação dos compostos, garante pouco solvente residual que estão de acordo com as normas para consumo humano e os produtos são obtidos em condições mais brandas de temperatura e pressão podendo ser utilizada em compostos termossensíveis (AGUIAR et al., 2016). Pesquisas evidenciam que a micronização de moléculas reduz o tamanho de partículas, gerando mudanças em estrutura física, aumento da solubilidade e taxa de dissolução, essas melhorias podem aumentar significativamente a biodisponibilidade e o potencial

terapêutico de compostos (AGUIAR et al., 2016, 2017, 2018; ALMEIDA et al., 2021; BERTONCELLO et al., 2018; DECUI et al., 2020).

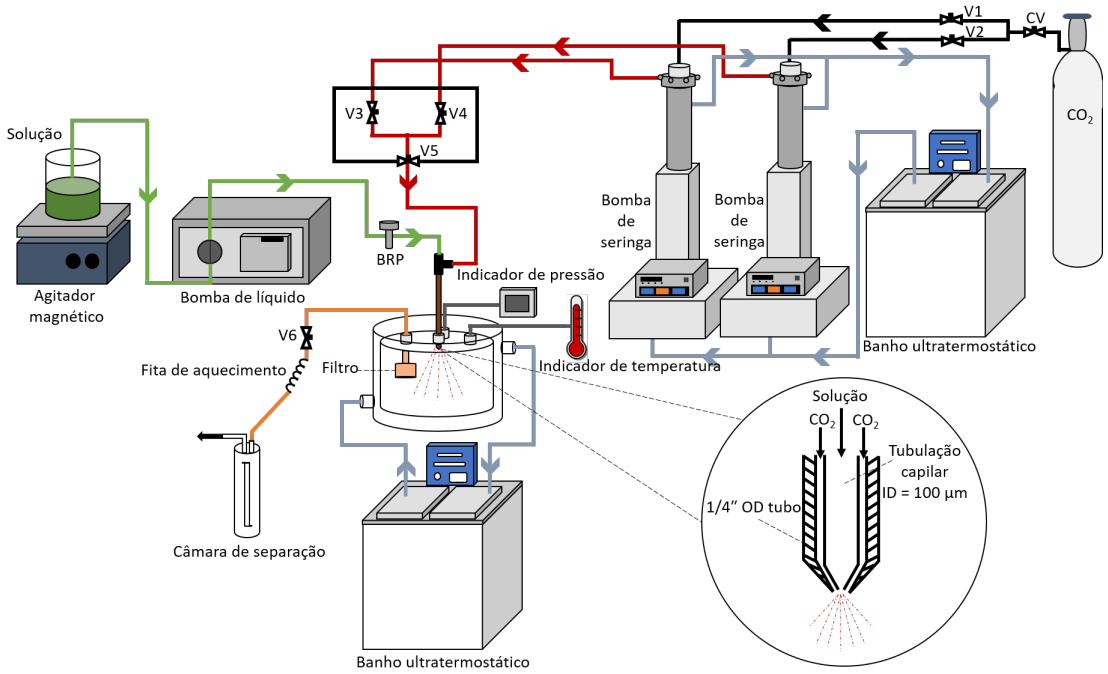

Figura 5. Diagrama esquemático do aparato experimental usando na técnica de dispersão de solução aumentada por fluidos supercríticos (SEDS). CV (válvula de retenção), V1, V2, V3 e V4 (válvula esférica), V5 e V6 (válvula de agulha) e BRP (regulador de contrapressão).

Estudos também demonstraram que a micronização reduziu o tamanho de partícula das saponinas presentes em *Panax notoginseng,* alterando a dissolução/liberação e aumentando a concentração no plasma de ratos em comparação com as preparações de tamanho de partícula maior (LIANG et al., 2021). Além disso, a fração flavonóide purificada e micronizada (diosmina + hesperidina) mostrou um aumento do efeito anticoagulante em ratos (MCGREGOR et al., 1999). Em humanos, o resveratrol micronizado (SRT501) apresentou maior concentração plasmática, proporcionando níveis mensuráveis de resveratrol no tecido hepático de pacientes com câncer colorretal e metástases hepáticas. Além disso, STR501 aumentou a caspase-3 clivada (um biomarcador de apoptose) no tecido hepático maligno (HOWELLS et al., 2011). Nosso grupo mostrou que a micronização por SEDS diminuiu a concentração efetiva mínima de N-acetilcisteína (NAC) necessária para o efeito ansiolítico no peixe-

zebra (AGUIAR et al., 2017). Além disso, a curcumina micronizada e o resveratrol, mas não os compostos não micronizados, mostraram efeitos semelhantes aos antiepilépticos diazepam e valproato de sódio, diminuindo a frequência e a gravidade de crises epilépticas agudas induzida por pentilenotetrazol em peixes-zebra (ALMEIDA et al., 2021; BERTONCELLO et al., 2018; DECUI et al., 2020).

2. OBJETIVOS

2.1 Objetivos gerais

Comparar os efeitos da curcumina convencional (CUR) e micronizada (CM) sobre atividade antioxidante *in vitro* e em parâmetros comportamentais e bioquímicos em peixes-zebra.

2.2 Objetivos específicos

Comparar os efeitos da curcumina convencional (CUR) e micronizada (CM) sobre:

2.2.1. Atividade antioxidantes *in vitro* pelos testes de FRAP (poder antioxidante redutor de ferro), DPPH (remoção do radical 1,1-difenil-2-2-piciril-hidrazil), GSH (proteção contra a oxidação da glutationa) e desoxirribose (inibição da formação do radical hidroxila);
2.2.2. Parâmetros comportamentais nos testes de interação social (SI), tanque novo (NTT) e tanque aberto (OTT) e parâmetros bioquímicos através dos níveis de tióis não proteicos (NPSH), atividade das enzimas antioxidantes glutationa redutase (GR) e glutationa peroxidase (GPx) e níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS) em peixes-zebra submetidos ao modelo de estresse crônico imprevisível (ECI);
2.2.3. Parâmetros comportamentais no teste de tanque aberto (OTT) e parâmetros bioquímicos através dos níveis de tióis não proteicos (NPSH) e substâncias reativas ao ácido tiobarbitúrico (TBARS) em peixes-zebra submetidos ao modelo de estresse agudo por contenção (EAC);
2.2.4. Parâmetros comportamentais no teste de tanque aberto (OTT), claro/escuro (LDT) e estímulo aversivo (AST) e parâmetros bioquímicos pelos níveis de tióis não proteicos

(NPSH) e substâncias reativas ao ácido tiobarbitúrico (TBARS) em larvas de peixes-zebra.

3. COLETÂNEA DE ARTIGOS

A presente tese é apresentada no formato de coletânea de artigos científicos submetidos a periódicos de acordo com cada objetivo específico.

3.1. CAPÍTULO I: Curcumin micronization by supercritical fluid: in vitro and in vivo biological relevance.

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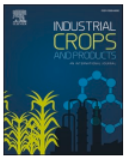

Curcumin micronization by supercritical fluid: In vitro and in vivo biological relevance

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Curcumin micronization by supercritical fluid: *in vitro* and *in vivo* biological relevance

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ABSTRACT

Curcumin, a polyphenol extracted from the rhizome of *Curcuma longa* L. (Zingiberaceae), is shown to have antioxidant, anti-inflammatory, neuroprotective, anxiolytic, and antidepressant properties in both preclinical and clinical studies. However, its low bioavailability is a limitation for its potential adoption as a therapeutic agent. The process of micronization can overcome this barrier by reducing the particle size and increasing the dissolution rate, potentially improving the bioavailability of the compounds of interest. In this study, we compared the *in vitro* antioxidant effects of curcumin (CUR) and micronized curcumin (MC) and studied their effects on behavioral and neurochemical parameters in zebrafish submitted to unpredictable chronic stress (UCS). MC (1 g/L) presented higher antioxidant activity *in vitro* as compared to CUR, as measured by iron-reducing antioxidant power (FRAP), 1,1-diphenyl-2-2-picyryl-hydrazyl radical removal (DPPH), and deoxyribose tests. UCS increased total distance traveled in the social interaction test (SI), while decreased crossings, time, and entries to the top area in the novel tank test (NTT). No effects of UCS were observed in the open tank test (OTT). The behavioral effects induced by UCS were not blocked by any curcumin preparation. UCS also decreased non-protein thiols (NPSH) levels, while increased glutathione reductase (GR) activity and thiobarbituric acid reactive substances (TBARS) levels on zebrafish brain. MC presented superior antioxidant properties than CUR *in vivo*, blocking the stress-induced neurochemical effects. Although this study did not measure the concentration of curcumin on the zebrafish brain, our results suggest that micronization increases the bioavailability of curcumin, potentiating its antioxidant activity both *in vitro* and *in vivo*. Our study also demonstrates that counteracting the oxidative imbalance induced by UCS is not sufficient to block its behavioral effects.

Keywords: supercritical fluids, unpredictable chronic stress, oxidative damage, antioxidant, curcumin, micronization, zebrafish.

1. Introduction

Stress is an adaptative process by which the body reacts to an external stimulus or threat. Physiological, behavioral, and metabolic adaptations through the activation of the sympathetic autonomic nervous system and the hypothalamic-pituitary-adrenal axis (HPA) are triggered so that an organism adequately responds to that stimulus (Kaufmann et al., 2016; Mcewen, 2006; McEwen et al., 2015). However, when the individual is exposed to chronic stress, depletion of the adaptive response can occur with deleterious results. In this case, hyperactivation of the sympathetic autonomic nervous system and HPA axis, increased cortisol levels, dysfunction of neurotransmitter systems, neuroinflammation, defects in neurogenesis and synaptic plasticity, mitochondrial dysfunction, redox state imbalance, and oxidative damage may be present. These complex neurobiological changes can predispose the individual to mental disorders such as anxiety and depression (Cernackova et al., 2020; Popoli et al., 2012). The neurobiological basis of mental disorders is not fully understood, but studies have already shown that oxidative status imbalances are present in animal models and humans (Avery, 2011; Fedoce et al., 2018; Harwell, 2007; Morris et al., 2020; Picard et al., 2018; Valko et al., 2007). Therefore, compounds with antioxidant activity are potential candidates, as a complement to the non-pharmacological and pharmacological therapeutic approaches.

Curcumin is one of the compounds extracted from *Curcuma longa* L. (Zingiberaceae) roots, widely used in Asian countries as a food coloring and seasoning component. Several works have demonstrated the potent antioxidant activity of this compound, both *in vitro* and *in vivo* (Ak and Gülçin, 2008; Menon and Sudheer, 2007). Curcumin can attenuate intracellular production of reactive oxygen species (ROS), increase antioxidant enzyme activity, and protect mitochondria from oxidative damage in rats (Wei et al., 2006; Zhu et al., 2016). Moreover, this compound has shown anti-inflammatory, neuroprotective, and immunomodulatory effects (Bhutani et al., 2009; Kulkarni et al., 2008; Reeta et al., 2010; Wang et al., 2008, 2014; Yadav et al., 2005; Zhao et al., 2014).

However, curcumin has low bioavailability due to poor absorption, rapid metabolism, and quick systemic elimination, which compromise its therapeutic use for neuropsychiatric disorders (Anand et al., 2007; Yang et al., 2005). The micronization process reduces the size of particles modifying the conformation of crystal structure, changing the physical structure, and enhancing solubility and dissolution rates. As expected, these effects could lead to an improvement in the bioavailability of curcumin.

Previous studies have shown that micronization reduced the particle size of *Panax notoginseng* saponins, changing dissolution/release and increasing concentration in the plasma of rats compared with the larger particle size preparations (Liang et al., 2021). Also, the micronized purified flavonoid fraction (diosmin + hesperidin) showed a significant anticoagulant effect, increasing platelet disaggregation in rats (McGregor et al., 1999). In humans, micronized resveratrol (SRT501) showed higher plasmatic concentration, providing measurable resveratrol levels in liver tissue of patients with colorectal cancer and hepatic metastases. Moreover, STR501 increased the cleaved caspase-3 (an apoptosis biomarker) in malignant hepatic tissue (Howells et al., 2011). Our group has shown that micronization decreased the minimum effective concentration of N-acetylcysteine (NAC) required to exert an anxiolytic effect in zebrafish (Aguiar et al., 2017). Also, micronized curcumin and resveratrol, but not non-micronized compounds, showed similar effects as the antiepileptic drugs diazepam and valproate, reducing seizure occurrence and slowing seizure progression in the PTZ-induced seizure model in zebrafish (Almeida et al., 2021; Bertoncello et al., 2018; Decui et al., 2020).

Considering the role of chronic stress in neuropsychiatric disorders and the potential neuromodulatory effects of curcumin, this study aimed to compare the effects of curcumin and micronized curcumin on behavioral and neurochemical parameters in adult zebrafish submitted to the unpredictable chronic stress. In addition, we compared the antioxidant effects of both preparations *in vitro*.

2. Materials and methods

2.1 Drugs

Curcumin was obtained from Sigma-Aldrich® (CAS 458-37-7) (St. Louis, MO, USA), and its micronization was carried out at the Laboratory of Thermodynamics and Supercritical Technology (LATESC) of the Department of Chemical and Food Engineering (EQA) at UFSC. Both curcumins preparations were dissolved in 1% DMSO (Dimethyl sulfoxide anhydrous) obtained from Sigma-Aldrich® (CAS 67-68-5) and diluted in injection water (Samtec biotecnologia®, SP, Brazil) acquired from a commercial supplier. Other reagents for neurochemical analysis were obtained from Sigma-Aldrich®.

2.2 Curcumin micronization with the solution enhanced dispersion by supercritical fluids (SEDS)

The SEDS experimental equipment and procedure used to micronize curcumin with supercritical carbon dioxide (CO_2) as an anti-solvent was described in detail in previous studies (Dal Magro et al., 2017; Machado et al., 2014). A schematic diagram of the experimental apparatus is presented in Figure 1. The process parameters adopted in the present report were based on previous data: solute concentration of 20 mg/mL, temperature at 35 °C, anti-solvent flow rate of 20 mL/min, solution flow rate of 1 $mL \cdot min^{-1}$, and operating pressure of 8 MPa (Aguiar et al., 2018, 2017, 2016; Bertoncello et al., 2018).

2.3 Morphology and determination of particle size

CUR and MC samples were submitted to morphological analysis by Scanning Electron Microscopy (SEM) (JEOL JSM-6390LV United States), with 10 kV power and 300-500 objectives, to determine particle morphology and Meter Size software

(version 1.1) was used to determine the mean particle size (Aguiar et al., 2016; Bertoncello et al., 2018).

2.4 Differential scanning calorimetry

The melting point of the CUR and MC was determined using a system of differential scanning calorimetry (DSC) (Jade-DSC, Perkin Elmer). The samples (5–10 mg) were prepared in an aluminum pan, and DSC measurements were performed by heating at 30 to 200 °C at a rate of 10 °C/min in an inert atmosphere (N_2 flow: 10 mL/min) (Aguiar et al., 2016; Bertoncello et al., 2018).

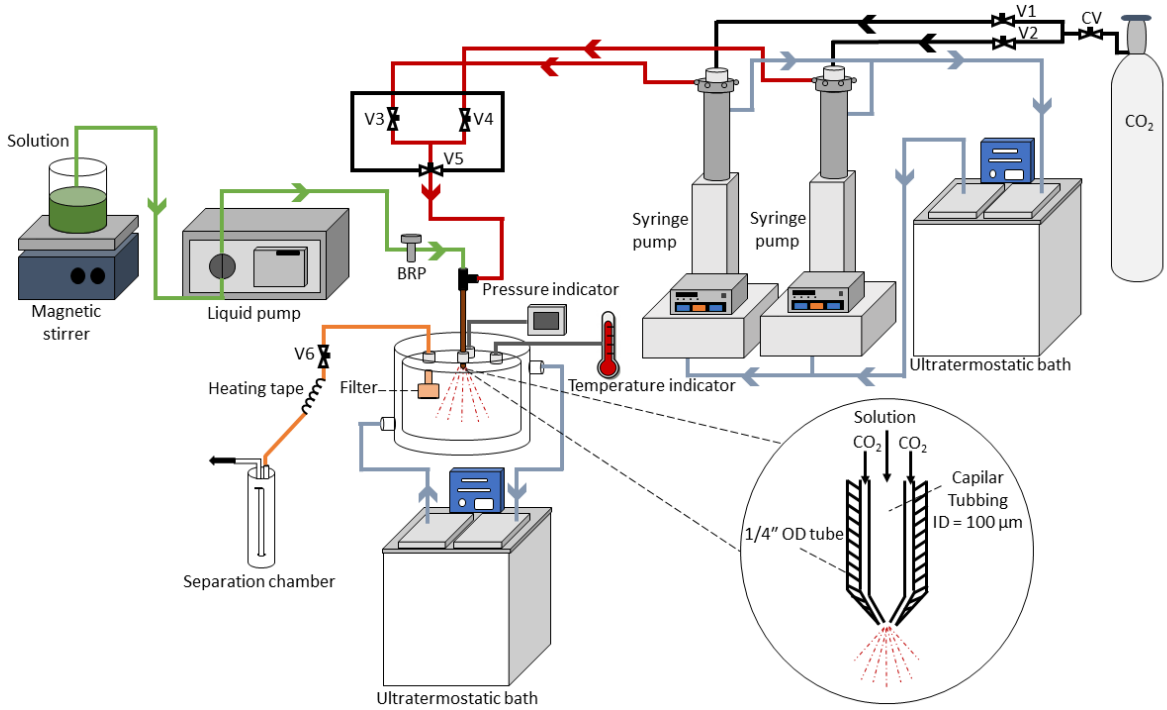


Fig. 1 Schematic diagram of the experimental apparatus using the solution enhanced dispersion by supercritical fluids technique (SEDS). CV (check valve), V1, V2, V3, and V4 (ball valve), V5 and V6 (needle valve), and BRP (backpressure regulator).

2.5 In vitro antioxidant activity

To analyze the antioxidant activity *in vitro*, the following experimental groups were tested: 1% DMSO; ascorbic acid (0.0625, 0.25 and 1 g/L, as positive control); CUR (0.0625, 0.25 and 1 g/L) and MC (0.0625, 0.25 and 1 g/L). These concentrations were based on previous studies (Bertoncello et al., 2018; Gilhotra and Dhingra, 2010; Xu et al., 2005). All analyses were performed in duplicate with an n=5. A schematic diagram of the experimental tests is presented in Figure 2.

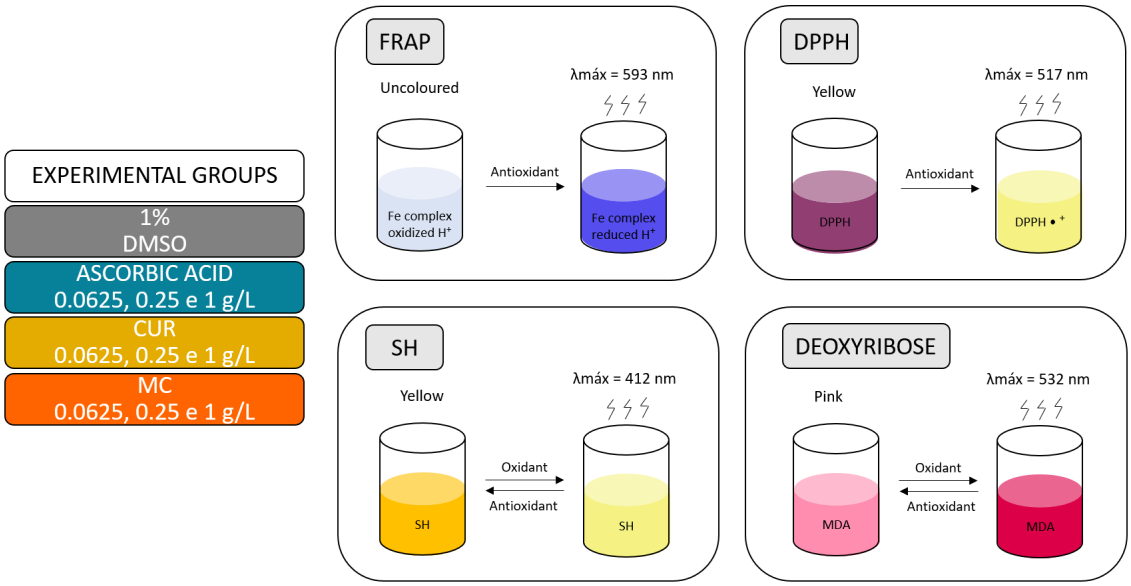


Fig. 2 Experimental design of the *in vitro* assays: FRAP (iron-reducing antioxidant power), DPPH (1,1-diphenyl-2-2-picyryl-hydrazyl radical removal), GSH (protection against glutathione oxidation*)*, deoxyribose assay. DMSO (dimethyl sulfoxide), CUR (curcumin) and MC (micronized curcumin).

2.5.1 Determination of iron-reducing antioxidant power (FRAP)

The antioxidant power was assessed by the reduction of Fe^{3+} to Fe^{2+}. The samples were incubated at 37 °C for 15 min with a reaction medium containing: 10 mM 2,4,6-Tripyridyl-Triazine (TPTZ) + 40 mM hydrochloric acid (HCl), $FeCl_3$. 20 mM $6H_2O$ and 300 mM acetate buffer (10:1:1 ratio). The absorbance was read at 593

nm. Blanks for each sample were incubated without TPTZ (Benzie and Strain, 1996). The detailed protocol is available at protocols.io (Sachett et al., 2021a).

2.5.2 1,1-diphenyl-2-2-picyryl-hydrazyl radical removal test (DPPH)

The scavenging capacity of free radicals was assessed by the DPPH assay. The samples at different concentrations were incubated for 24 h in the dark with methanol and DPPH (0.24 mg/mL). DPPH and methanol, without samples, were used as controls. Methanol was used as a blank. After incubation, the absorbance was read at 517 nm. The effective concentration 50 (EC50) for each extract, which expresses the minimum amount of the extract capable of reducing the initial concentration of DPPH radical by 50%, was calculated by non-linear regression using GraphPad Prism version 8 (Brand-Williams et al., 1995). The detailed protocol is available at protocols.io (Sachett et al., 2021b).

2.5.3 Protection against glutathione oxidation (GSH)

To quantify the presence of sulfhydryl groups after oxidation induced by hydrogen peroxide (H_2O_2). Each sample was incubated for 30 min in the dark, with a reaction medium containing potassium phosphate buffer (TFK) (200 mM, pH 6.4) and H_2O_2 (5 mM). Afterward, the mixture was added to 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (10 mM) and the absorbance was read at 412 nm after 5 min. Samples without GSH were used as sample blank and the incubation medium without sample was used as a control (Ellman, 1959). The detailed protocol is available at protocols.io (Sachett et al., 2021c).

2.5.4 Deoxyribose assay

The production of malondialdehyde (MDA) after oxidation of deoxyribose by hydroxyl radical (OH·) was measured through the TBARS. The samples were

incubated at 37°C for 1 h, with a reaction medium containing: KH_2PO_4-KOH (50 mM, pH 7.4), deoxyribose (60 mM), $FeCl_3$ (1 mM), ethylenediaminetetraacetic acid (EDTA) (1.04 mM), ascorbic acid (2 mM), and H_2O_2 (10 mM). Then, 1% thiobarbituric acid (TBA) and 25% HCl were added to the mix and heated in a water bath at 100 °C for 15 min. The absorbance was read at 532 nm. Samples without deoxyribose were used as sample blank and the incubation medium without sample was used as a control (Halliwell et al., 1987). The detailed protocol is available at protocols.io (Sachett et al., 2021d).

2.6. Animals

All procedures were approved by the institutional animal welfare and ethical review committee at the Federal University of Rio Grande do Sul (UFRGS) (approval #35279/2018). The animal experiments are reported in compliance with the ARRIVE guidelines 2.0 (Percie du Sert et al., 2020). Experiments were performed using 108 male and female (50:50 ratio) short-fin wild-type zebrafish, 6 months old, weighing 300 to 400 mg. Adult animals were obtained from the colony established at the Biochemistry Department of UFRGS and maintained in our animal facility (Altamar, SP, Brazil) in a light/dark cycle of 14/10 hours for at least 15 days before tests. Fish were transferred to 16-L home tanks (40 x 20 x 24 cm) with non-chlorinated water kept under constant mechanical, biological, and chemical filtration at a maximum density of two animals per liter. Tank water satisfied the controlled conditions required for the species (26 ± 2 °C; pH 7.0 ± 0.3; dissolved oxygen at 7.0 ± 0.4 mg/L; total ammonia at <0.01 mg/L; total hardness at 5.8 mg/L; alkalinity at 22 mg/L $CaCO_3$; and conductivity of 1500–1600 μS/cm). Food was provided twice a day (commercial flake food (Poytara®, Brazil) plus the brine shrimp *Artemia salina*).

The animals were allocated to the experimental groups following block randomization procedures to counterbalance the sex, the two different home tanks, and the test arenas between the groups. Each experimental group was originated from two identical home tanks. Animal behavior was video recorded and analyzed with the

ANY-Maze tracking software (Stoelting Co., Wood Dale, IL, USA) by researchers blinded to the experimental groups. All tests were performed between 08:00 and 12:00 a.m. The sex of the animals was confirmed after euthanasia by dissecting and analyzing the gonads. For all experiments, no tank and sex effects were observed, so data were pooled together.

After the tests, animals were euthanized by hypothermic shock according to the AVMA Guidelines for the Euthanasia of Animals (Leary and Johnson, 2020). Briefly, animals were exposed to chilled water at a temperature between 2 and 4 °C for at least 2 min after loss of orientation and cessation of opercular movements, followed by decapitation as a second step to ensure death.

2.7 Drug administration

Intraperitoneal (i.p.) injections were applied using a Hamilton Microliter™ Syringe (701N 10 µL SYR 26s/2"/2) x Epidural catheter 0.45 x 0.85 mm (Perifix®-Katheter, Braun, Germany) x Gingival Needle 30G/0.3 x 21 mm (GN injecta, SP, Brazil). The injection volume was 1 µL/100 mg of animal weight. The animals were anesthetized by immersion in a solution of tricaine (300 mg/L, CAS number 886-86-2) until loss of motor coordination and reduction of respiratory rate. The anesthetized fish were gently placed in a sponge soaked in water placed inside a petri dish, with the abdomen facing up and the fish's head positioned on the sponge's hinge. The needle was inserted parallel to the spine in the abdomen's midline posterior to the pectoral fins. This procedure was conducted in approximately 10 seconds. The behavioral tests took place 24 hours after the last injection. Drug solutions were prepared daily.

2.8 Unpredictable chronic stress (UCS)

UCS was carried out based on previous studies (Bertelli et al., 2021; Marcon et al., 2019; Mocelin et al., 2019; Piato et al., 2011). The experimental design is presented in Figure 3 and the schedule and stressors are detailed in the supplementary

material (Table S1). Initially, fish were divided into control (non-stressed group, S-) and UCS (stressed group, S+). After seven days, the experimental groups were subdivided into DMSO (1% DMSO), CUR (10 mg/kg), and MC (10 mg/kg) (this dose was chosen based on the concentration with the best antioxidant effect *in vitro)*. The animals were anesthetized daily and injected at 2:00 p.m. (as described above) and then returned to the home tanks. The animals' weight was checked on the 1st, 7th, and 14th day and an average between the weights of each tank was used to calculate the injection volume. After the UCS, fish were submitted to the SI, NTT, and OTT, performed on the 15th, 16th, and 17th days, respectively. On the 15th and 16th days, after the behavioral tests, the animals were also injected with the corresponding treatments. On the 17th day, immediately after the OTT, fish were euthanized, and the brain was dissected and homogenized for the biochemical assays.

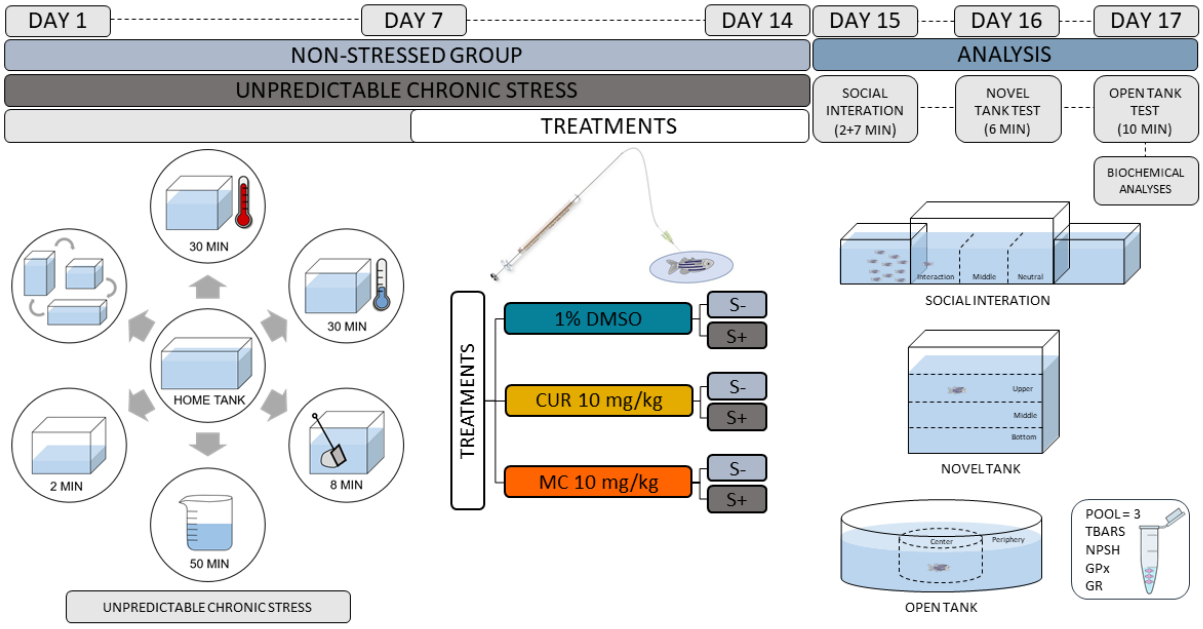


Fig. 3 Experimental design. Zebrafish remained in the home tank and were subjected to the UCS for 14 days or remained undisturbed (non-stressed control). After the first seven days of stress, zebrafish were daily injected i.p. with DMSO 1%, CUR 10 mg/kg, or MC 10 mg/kg. On the 15th of the experimental protocol, animals were subjected to the social interaction test. On the 16th animals were subjected to the novel tank test. On the 17th animals were subjected to the open tank test and then euthanized to collect the brain, which was used in biochemical analyses. DMSO (dimethyl sulfoxide), CUR (curcumin) and MC (micronized curcumin).

2.8.1 Social interaction test (SI)

The SI test was conducted as described previously (Benvenutti et al., 2020; Bertelli et al., 2021) Animals were placed individually for 7 min in a tank (30 x 10 x 15 cm, 10 cm water level) flanked by two identical tanks (15 x 10 x 13 cm, 10 cm water level), either empty (neutral stimulus) or containing 10 unfamiliar zebrafish (social stimulus) (Fig. 3). The position of the social stimulus (right or left) was counterbalanced throughout the tests. The test apparatus was virtually divided into three vertical areas (interaction, middle, and neutral). Videos were recorded from the front view. Animals were habituated to the apparatus for 2 min and then analyzed for 5 min. The following parameters were quantified: total distance traveled, number of crossings (transitions between the areas of the tank), time spent in the interaction area (as a proxy for social interaction time), and time spent in the neutral area (Seibt et al., 2011). We changed the water in the tanks between animals to avoid interference from drug traces or alarm substances released by previously tested fish.

2.8.2 Novel tank test (NTT)

The NTT was conducted as described previously (Benvenutti et al., 2020; Bertelli et al., 2021; Marcon et al., 2019; Mocelin et al., 2019). Animals were individually placed in the tank (24 × 8 × 20 cm, 15 cm water level) and recorded for 6 min. Videos were recorded from the front view. The test apparatus was virtually divided into three horizontal areas (top, middle, and bottom) (Marcon et al., 2019; Mocelin et al., 2019). The water in the tanks was changed between animals to avoid interference from drug traces or alarm substances released by previously tested fish. The following parameters were quantified: total distance traveled (m), number of crossings (transitions between the areas of the tank), time spent (s), and number of entries in the top area of the tank.

2.8.3 Open tank test (OTT)

The OTT was conducted as described previously (Benvenutti et al., 2020; Bertelli et al., 2021). Animals were individually placed in the center of a circular arena made of opaque white plastic (24 cm diameter, 8 cm walls, 2 cm water level) and recorded for 10 min. The apparatus was virtually divided into two areas for video analyses: the central area of 12 cm in diameter and the periphery. Videos were recorded from the top view. The following parameters were quantified: total distance traveled (m), number of crossings (transitions between the areas of the tank), absolute turn angle (°), and time spent in the center area of the tank (s) (Johnson and Hamilton, 2017; Krook et al., 2019)

2.9 Biochemical assays

For each independent sample, three brains were pooled (n=6) and homogenized in 450 µL of phosphate-buffered saline (PBS, pH 7.4, Sigma-Aldrich) and centrifuged at 10,000 g at 4 °C in a cooling centrifuge; the supernatants were collected and kept in microtubes on ice until the assays were performed. The detailed protocol for prepare brain tissue samples is available at protocols.io(Sachett et al., 2020a). The protein was quantified according to the Coomassie blue method using bovine serum albumin (Sigma-Aldrich) as a standard (Bradford, 1976). The detailed protocol for protein quantification is available at protocols.io (Sachett et al., 2020b)

2.9.1 Non-protein thiols (NPSH)

The content of NPSH in the samples was determined by mixing equal volumes of the brain tissue preparation (50 µg of proteins) and trichloroacetic acid (TCA, 6%), centrifuging the mix (10,000 g, 10 min at 4 °C), the supernatants were added to TFK (1 M) and DTNB (10 mM) and the absorbance was measured at 412 nm after 1 h. The detailed protocol is available at protocols.io (Sachett et al., 2020c).

2.9.2 Glutathione reductase activity (GR)

The GR activity in the samples was determined by mixing the sample (30 µg of protein) with a reaction medium containing TFK + EDTA (154 mM, pH 7.0) and nicotinamide adenine dinucleotide phosphate (NADPH, 2mM). Then, oxidized glutathione (GSSG, 20 mM) was added and the decrease of NADPH absorbance per minute was read at 340 nm. The detailed protocol is available at protocols.io (Sachett et al., 2021e)

2.9.3 Glutathione peroxidase activity (GPx)

The GPx activity in the samples was determined by mixing the sample (30 µg of protein) with a reaction medium containing TFK + EDTA (0.5 M, pH 7.0), NADPH (1.6 mM), GSH (10 mM), GR (2.5 U/mL), and 10 mM azide. Then, H_2O_2 (4 mM) was added and the decrease of NADPH absorbance per minute was read at 340 nm. The detailed protocol is available at protocols.io (Sachett et al., 2021f).

2.9.4 Substances reactive to thiobarbituric acid (TBARS)

The lipid peroxidation was evaluated by quantifying the production of TBARS. Samples (50 µg of proteins) were mixed with TBA (0.5%) and TCA (20%) (150 µL). The mixture was heated at 100 °C for 30 min. The absorbance of the samples was determined at 532 nm in a microplate reader. MDA (2 mM) was used as the standard. The detailed protocol is available at protocols.io (Sachett et al., 2020d).

2.10 Statistical analysis

We calculated the sample size to detect an effect size of 0.35 for the interaction between stress and treatment with a power of 0.9 and an alpha of 0.05 using G*Power 3.1.9.7 for Windows. The total distance traveled was defined as the primary outcome. The total sample size was 107, which was rounded up to 108 to yield n = 18 animals per experimental group.

The normality and homogeneity of variances were confirmed for all data sets using D'Agostino-Pearson and Levene tests, respectively. Results were analyzed by one-way (analysis of the antioxidant activity *in vitro),* or two-way (UCS) ANOVA followed by Tukey post hoc test when applicable. The outliers were defined using the ROUT statistical test and were removed from the analyses. This resulted in 6 outliers (1 animal from each group) removed from the SI test, 4 outliers (1 animal from CUR S-, MC S-, DMSO S+ and MC S+ groups) removed from the NTT and 2 outliers (1 animal from each DMSO S+ and MC S+ groups) removed from the OTT. The tank and sex effects were tested in all comparisons and no effect was observed, so the data were pooled.

Data are expressed as mean ± standard deviations of the mean (S.D.). The level of significance was set at $p<0.05$. Data were analyzed using IBM SPSS Statistics version 27 for Windows and the graphs were plotted using GraphPad Prism version 8.0.1 for Windows.

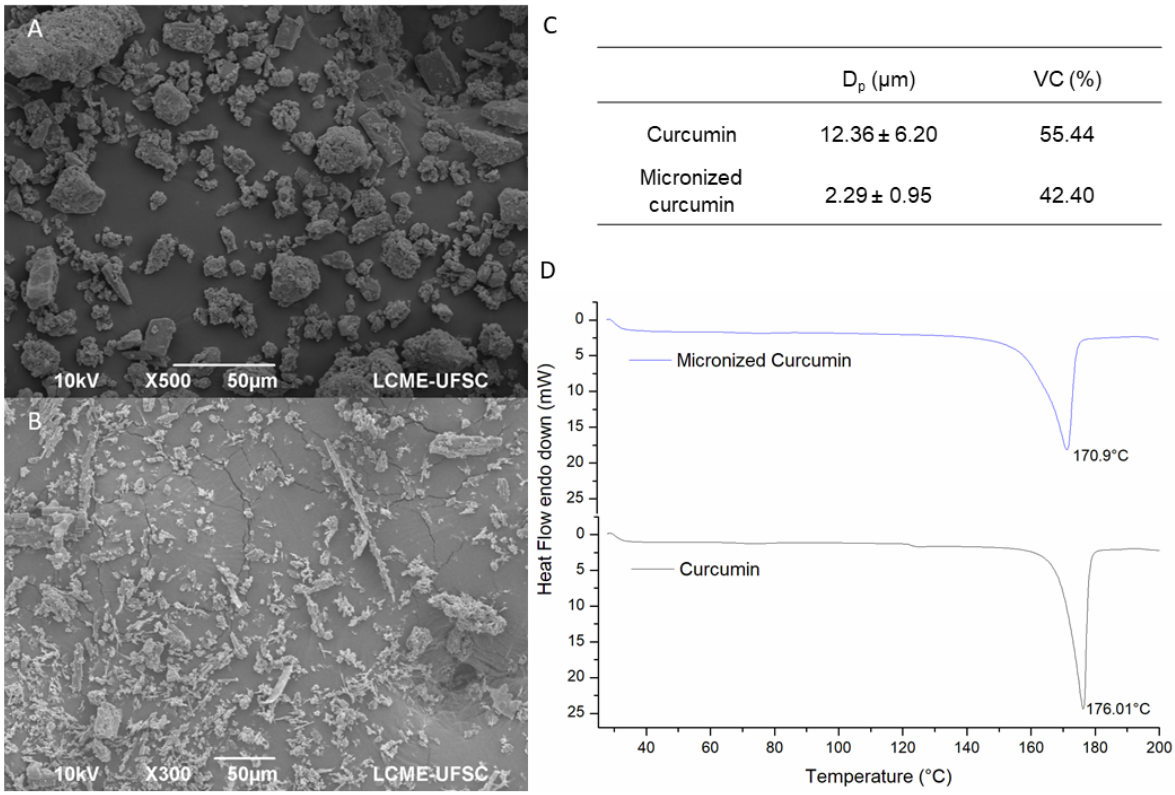
3. Results and discussion

3.1. Micronization

Characterization and size of the particles of the non-micronized and micronized curcumin are presented in Figure 4. The SEM showed a decrease in the size and change in the morphology of the CUR particles after micronization (Fig. 4A and 4B), being observed by the difference in zoom capable of visualizing the shape of the particles. CUR has an average size of 12.36 μm while the average size obtained through SEDS was 2.29 μm, which means a reduction of 5.4 times (Fig. 4C). The DSC

data (Fig. 4D) showed the detected melting point for curcumin was 176.01 °C, while for micronized curcumin the melting point was reduced to 170.9 °C. Changes to the melting point are related to alterations in dissolution and solubility rates, among other properties, and are caused by modification in the crystalline structure of the composts (Aguiar et al., 2018, 2017, 2016; Bertoncello et al., 2018; Chen et al., 2012; Cheng et al., 2016; Li et al., 2015; Moribe et al., 2005; Zhang et al., 2009).

The micronization of curcumin (Bertoncello et al., 2018), N-acetylcysteine (Aguiar et al., 2017), trans-resveratrol (Aguiar et al., 2018, 2016; Almeida et al., 2021; Decui et al., 2020), methotrexate (Chen et al., 2012), phenylbutazone (Moribe et al., 2005), *Panax notoginseng* saponins (Liang et al., 2021), etoposide (Cheng et al., 2016), ellagic acid (Li et al., 2015), taxifolin (Zu et al., 2012) atorvastatin calcium (Zhang et al., 2009) and ibuprofen (Han et al., 2011; Sosna et al., 2018) by the SEDS technique have shown a reduction in particle size, an increase in the dissolution rate, an increase in the solubility, as well as a modification of the crystalline structure of the compound when compared with non-micronized composts. Recently, the micronization of *Panax notoginseng* saponins changed the pharmacokinetic parameters of these compounds in rats, showing significantly higher values in plasma powders with smaller particle sizes than the larger particle sizes (Liang et al., 2021). Therefore, micronization can improve the bioavailability of compounds, being a critical tool for the industry, especially for compounds with low solubility (Aguiar et al., 2016; Chau et al., 2007; Chen et al., 2012; Li et al., 2015).



	D_p (µm)	VC (%)
Curcumin	12.36 ± 6.20	55.44
Micronized curcumin	2.29 ± 0.95	42.40

Fig. 4. Effects of micronization of curcumin on size values and thermal analysis. (A) particle size in the scanning electron microscope of non-micronized curcumin, (B) particle size in the scanning electron microscope of micronized curcumin, (C) particle size values in raw compounds, (D) differential scanning calorimetry. Dp (average particle diameter); VC (variation coefficient).

3.2. Antioxidant activity in vitro

The experimental design is summarized in Figure 2. In this experiment, we analyzed the *in vitro* antioxidant effects of CUR and MC in comparison to the positive control ascorbic acid. The FRAP method quantifies the electron-donating capacity of a compound by reduction of iron from ferric status to ferrous in solution (Halliwell, 1990). MC (at 0.25 and 1 g/L) increased the reduction of iron compared to CUR in the same concentrations ($p < 0.0001$, $F_{9,40} = 316.2$, Fig. 5A). As expected, ascorbic acid showed a greater reducing capacity than MC and CUR in all concentrations. MC and ascorbic acid in all concentrations and CUR at 0.25 and 1 g/L exhibited an increased

reduction potential when compared to 1% DMSO. These results indicate that the electron-donating capacity was increased by micronization. Similar results have been found in the literature where micronization increased the iron-reducing capacity (Li et al., 2015; Lu et al., 2020; Zhu et al., 2014).

DPPH is a free radical used to evaluate the antioxidant capacity through its elimination by reduction via donation of a hydrogen atom by a compound (Brand-Williams et al., 1995). In the DPPH assay, MC 1 g/L increased the scavenging of the DPPH radical when compared to CUR at the same concentration, being equivalent to the positive control ascorbic acid at all concentrations tested ($p<0.0001$, $F_{9,40} = 228.2$, Fig. 5B). CUR 0.0625 g/L removed more radicals than MC and ascorbic acid at the same concentration, but less than ascorbic acid at 1 g/L. MC, CUR, and ascorbic acid, in all concentrations tested, showed more inhibition of the radical than DMSO. There was no statistical difference in the EC_{50} among the treatments. Other studies reported that resveratrol, NAC, taxifolin, ellagic acid, and apple pomace micronized by SEDS, increased the antioxidant activity when compared to the non-micronized compost in DPPH tests (Aguiar et al., 2018, 2017; Li et al., 2015; Lu et al., 2020; Zu et al., 2012).

There were no significant differences between treatments on the percentage of remaining sulfhydryl groups of GSH (Fig. 5C). However, all treatments were able to inhibit oxidation of GSH measured by the remaining sulfhydryl groups of GSH that react with DTNB after oxidation induced by H_2O_2.

In the deoxyribose assay, OH· generated by Fenton reaction, oxidizes deoxyribose with the formation of MDA, quantified by TBARS (Halliwell et al., 1987). Thus, the ability of the compound to inhibit the formation of OH· and, consequently, the inhibition of the formation of MDA was analyzed. Both MC and CUR 1 g/L increased the inhibition of OH· production when compared to ascorbic acid ($p <0.0001$, $F_{9,40} = 13.92$, Fig. 5D). However, ascorbic acid 0.0625 g/L showed a greater inhibition when compared to CUR and MC at the same concentration and, also ascorbic at 2.5 g/L when compared to MC 2.5 g/L. MC 0.25 g/L showed less inhibition than 1% DMSO.

These results indicate that curcumin can eliminate peroxides, inhibit the formation of free radicals, and protect against an imbalance in the oxidative state of the organism and lipid peroxidation. Studies indicate that the antioxidant effect of curcumin can be attributed to its phenolic groups, acting as reducing agents, hydrogen donors, as well as oxygen adsorption inhibitors (Zheng et al., 2017). This supports our results suggesting that the ability to eliminate free radicals from MC obtained by the DPPH and deoxyribose assay is related to their reducing properties in the FRAP. Similar results have already been observed in several studies using curcumin preparations *in vitro* (Landeros et al., 2017; Sahu, 2016; Singh et al., 2018). However, this is the first study evaluating these effects in micronized curcumin.

Studies using this technology showed that the SEDS process is a robust methodology for improving the physicochemical properties and antioxidant activity by increasing the bioaccessibility and availability of phenolic and other compounds active (Lu et al., 2020; Sefrin Speroni et al., 2021; Zhou et al., 2004; Zu et al., 2012). Furthermore, positive correlations were detected between radical scavenging activity, ferric reducing antioxidant power, and total phenolic content of wine grape pomace micronized (Zhu et al., 2014). These data suggest the possibility of improving the antioxidant effects of composts by micronization.

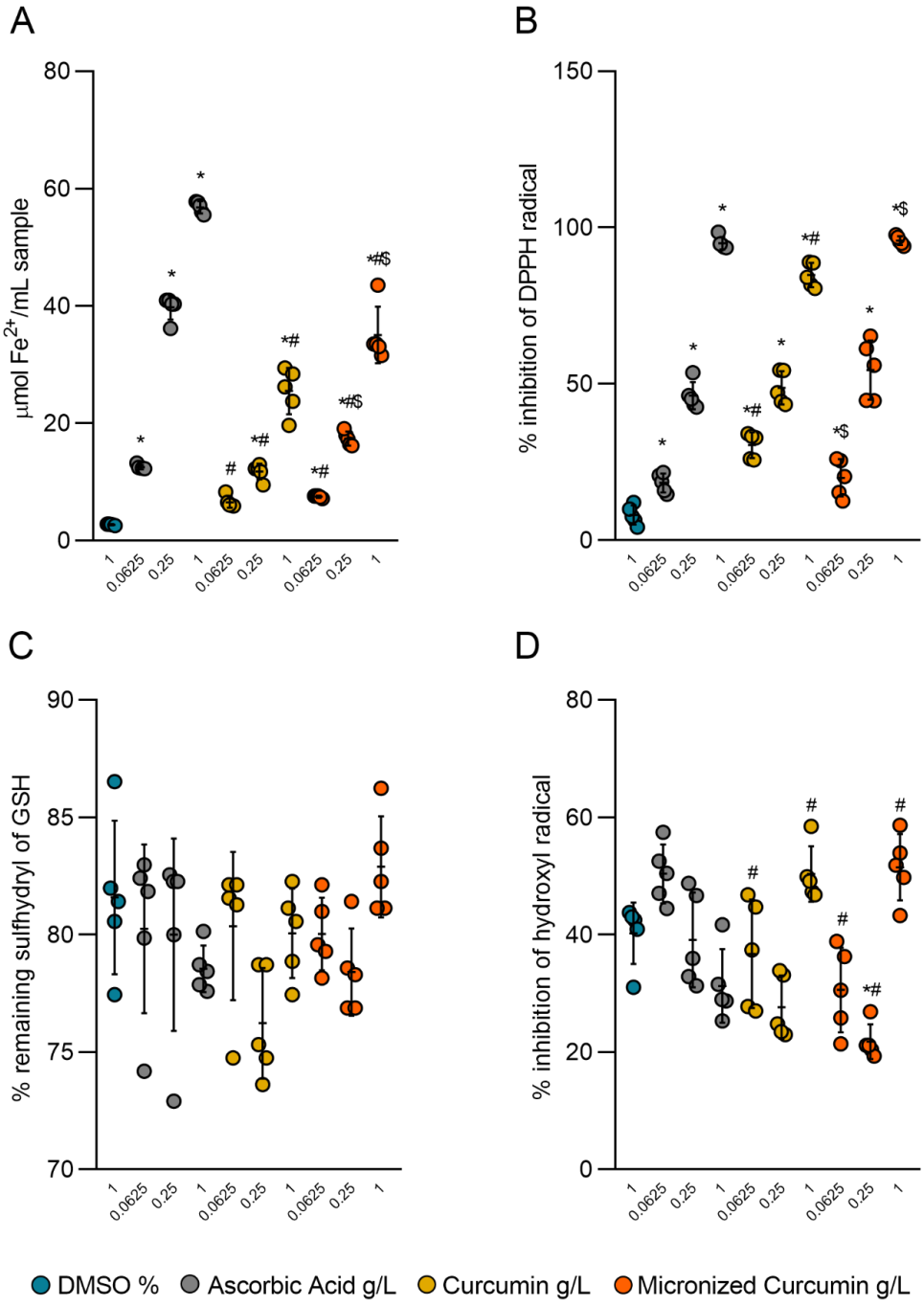


Fig. 5. Effects of DMSO, Ascorbic Acid, Curcumin, and Micronized curcumin on antioxidant activity *in vitro*. Data are expressed as mean ± S.D. One-way ANOVA/Tukey. n= 5. *p<0.05 x 1% DMSO. #p<0.05 x ascorbic acid (in the same concentration). $p<0.05 x curcumin (in the same concentration). DMSO (dimethyl sulfoxide), FRAP (ferric reducing antioxidant power), DPPH (1,1-diphenyl-2-2-picyryl-hydrazyl), and GSH (L-Glutathione reduced).

3.3 Effects of CUR and MC on behavioral and neurochemical parameters in zebrafish submitted to unpredictable chronic stress (UCS)

The timeline and experimental design are shown in Figure 3. Based on the most effective concentration of curcumin (1 g/L) observed in the *in vitro* antioxidant activity assays, we performed the UCS to verify the effects of both preparations on behavioral and neurochemical parameters in zebrafish. The unpredictable chronic stress induces several behavioral and neurochemical characteristics that resemble those observed in patients with anxiety and/or mood disorders (Chattarji et al., 2015; Willner, 2017, 2005). Antidepressants (Demin et al., 2020; Marcon et al., 2016; Reddy et al., 2021; Song et al., 2018), anxiolytics (Marcon et al., 2016), antioxidant (Marcon et al., 2019; Mocelin et al., 2019), ketamine (Reddy et al., 2021) and prazosin (O'Daniel and Petrunich-Rutherford, 2020) showed protective effects in this model.

Zebrafish live in social groups and, like humans, respond to the social support of conspecifics from a familiar shoal and recover from stressful events better in the presence of conspecifics (Faustino et al., 2017). In the SI test, two-way ANOVA revealed a main effect of stress on total distance (Fig. 6A), indicating a locomotor dysfunction induced by UCS. Both CUR and MC were unable to block this effect. The number of crossings (Fig. 6B), time in the interaction (Fig. 6C), and neutral (Fig. 6D) areas were not affected by UCS, indicating that, in this experimental condition, there was no impact of stress on social preference parameters. Notably, in the scientific literature, the effect of UCS on zebrafish shoaling behavior has been inconsistent, presenting decreased or increased social interaction after UCS (Chakravarty et al., 2013; Piato et al., 2011). In contrast to these studies, Fulcher et al. (2017) and Bertelli et al., (2021) found that UCS did not alter the social interaction of zebrafish in the social interaction test.

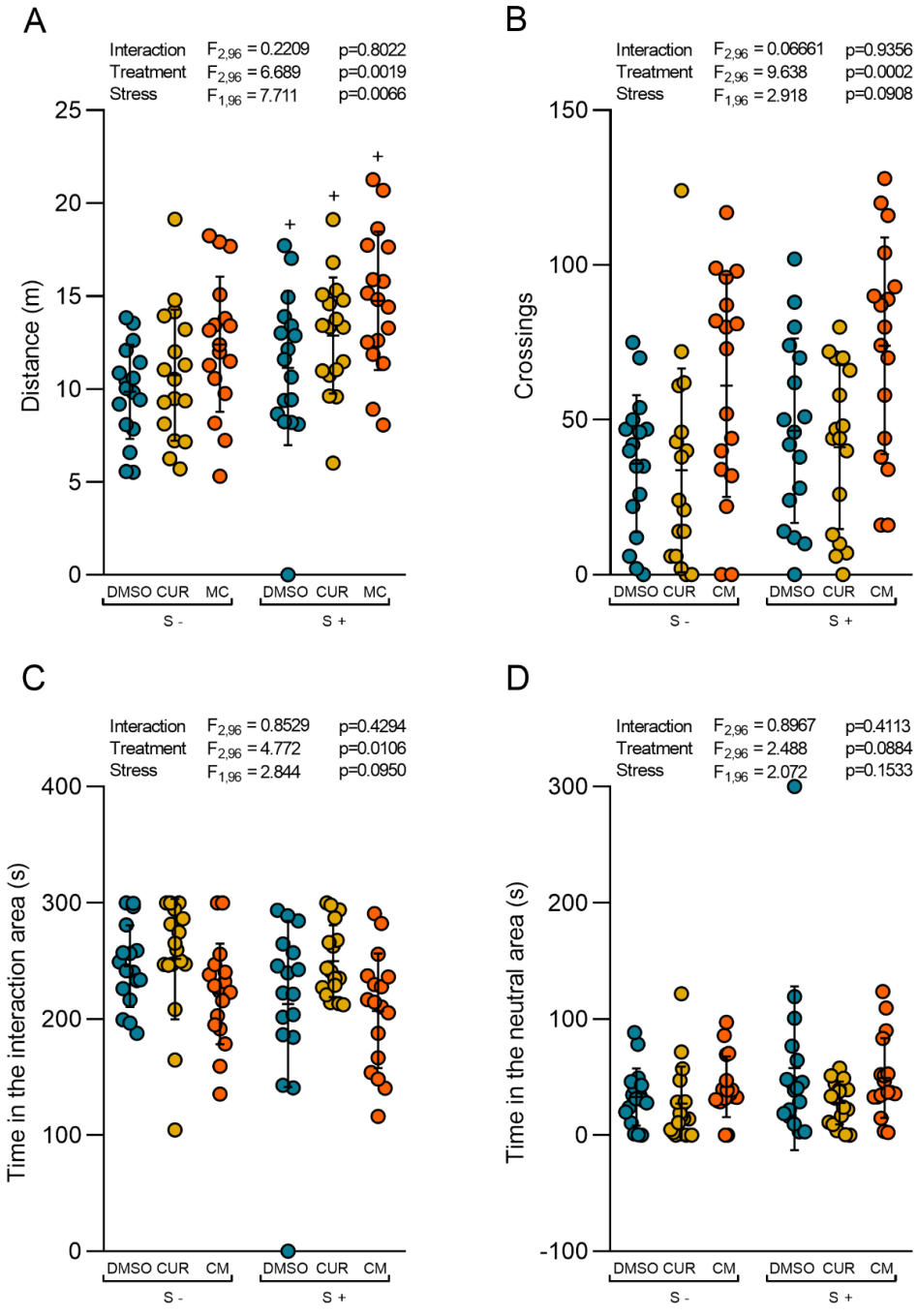


Fig. 6. Effects of CUR and MC (10 mg/kg) in zebrafish submitted to the UCS on the social interaction test (day 15). (A) total distance traveled (B) the number of crossings, (C) time in the interaction area and (D) time in the neutral area. Data are expressed as mean ± S.D. Two-way ANOVA/Tukey. n=17 $^{+}p<0.05$ stress effect. DMSO (dimethyl sulfoxide); CUR (curcumin); MC (micronized curcumin).

In the NTT, a reduction of the exploratory behavior towards the top area can be interpreted as an index of anxiety. Anxiolytic drugs such as fluoxetine, buspirone, and diazepam increase, whereas anxiogenic drugs such as caffeine and nicotine decrease the time on the top area of the tank (Bencan et al., 2009; Egan et al., 2009; Gebauer et al., 2011; Levin et al., 2007). Here, UCS decreased the number of crossings (Fig. 7B), time spent (Fig. 7C), and entries (Fig. 7D) to the top area of the tank in the NTT. Neither the UCS nor the treatments altered the total distance traveled (Fig. 7A). These results replicate and reinforce previous studies showing that UCS induces anxiety-like behavior in zebrafish (Chakravarty et al., 2013; Demin et al., 2020; Marcon et al., 2019, 2018a, 2016; Mocelin et al., 2019; O'Daniel and Petrunich-Rutherford, 2020; Piato et al., 2011; Reddy et al., 2021; Song et al., 2018). Both CUR and MC were unable to block these alterations.

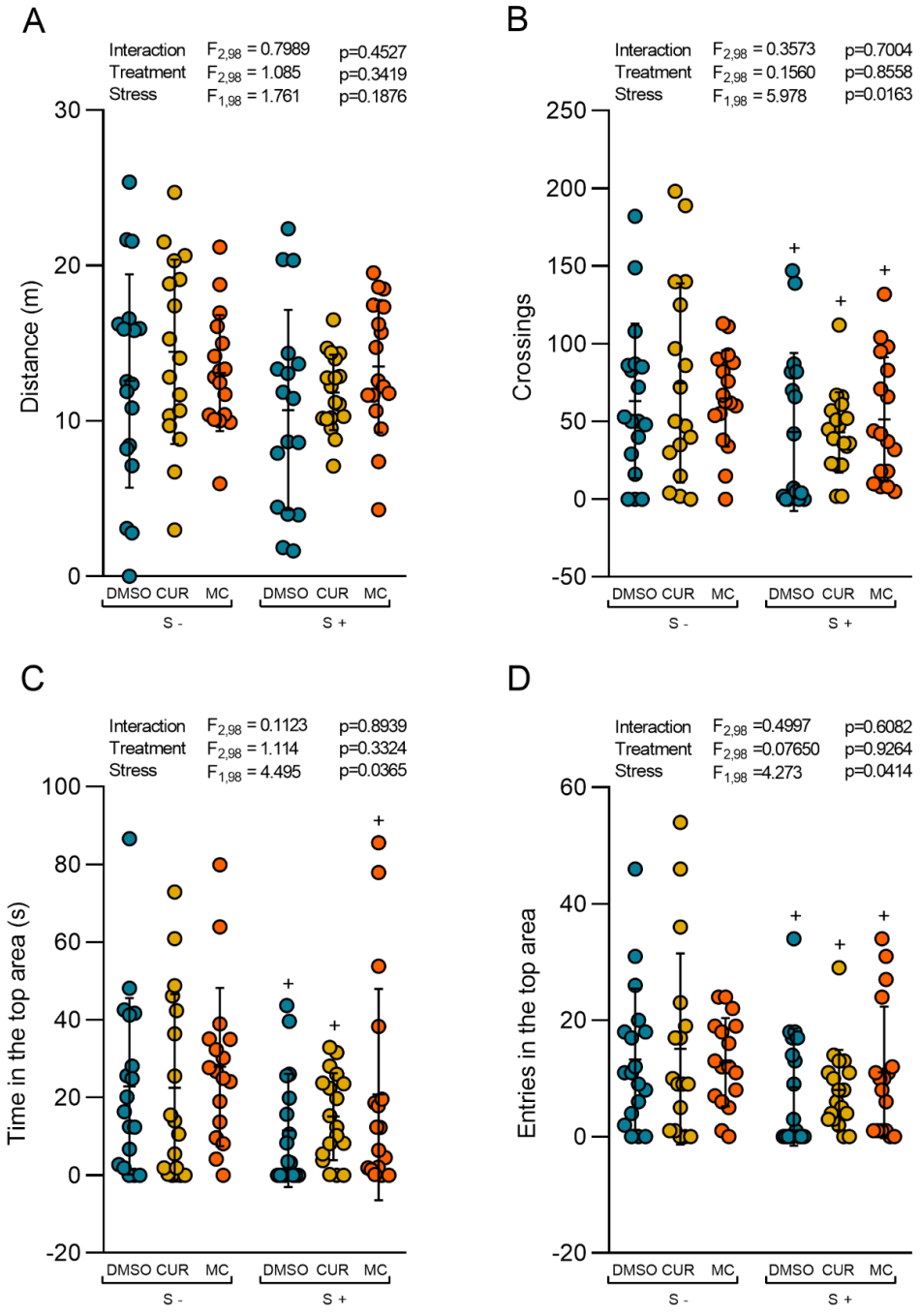


Fig. 7. Effects of CUR and MC (10 mg/kg) in zebrafish submitted to the UCS on the novel tank test (day 16). (A) total distance traveled, (B) the number of crossings, (C) time in the top area, (D) entries in the top area. Data are expressed as mean ± S.D. Two-way ANOVA/Tukey. n=17-18. $^{+}p<0.05$ stress effect. DMSO (dimethyl sulfoxide); CUR (curcumin); MC (micronized curcumin).

The OTT is a paradigm adapted from the open-field test (OFT) used in rodents, showing similarity with exploration, thigmotaxis, and freezing parameters. A decrease in the time spent in the thigmotaxis area (periphery) and an increase in exploration indicates a decrease in anxiety in zebrafish (Johnson and Hamilton, 2017; Stewart et al., 2012). Two-way ANOVA revealed an interaction between both factors on the number of line crossings (Fig. 8B), however, no significant effects were observed in *post hoc* analysis. In addition, there were no significant effects of any intervention on total distance, absolute turn angle, and time spent in the center area (Fig. 8A, 8C, and 8D, respectively).

Several studies evaluated the effects of curcumin using animal models of chronic stress. In rodents submitted to stress for 21 or 28 days, curcumin (10-40 mg/kg, i.p. or p.o. for the same time) blocked the stress effects decreasing immobility time in the OFT and memory deficits in the Morris water maze (MWM). In these studies, curcumin decreased serum corticosterone and increased BDNF and monoamine levels. Moreover, curcumin increased hippocampal neurogenesis and decreased brain monoamine oxidase activity, when compared to the stressed group (Bhutani et al., 2009; da Silva Marques et al., 2021; Xu et al., 2009, 2007, 2006). In rats submitted to UCS for 35 days, curcumin (40 mg/kg i.p. for 35 days) decreased the immobility time and increased the swimming time in the forced swim test, besides increased the percent of sucrose consumption (Fan et al., 2019, 2018). Similarly, treatment with curcumin (20 mg/kg p.o.) for 8 days increased the glucose preference and locomotor activity in OFT, as well as a decrease in the escape latency in MWM and the brain cytokines levels in rats submitted to UCS for 8 days (Vasileva et al., 2018). We supposed that the effects of curcumin on stress-induced behavioral changes might be observed with a longer exposure time or even in a dose range different from that used in the present study.

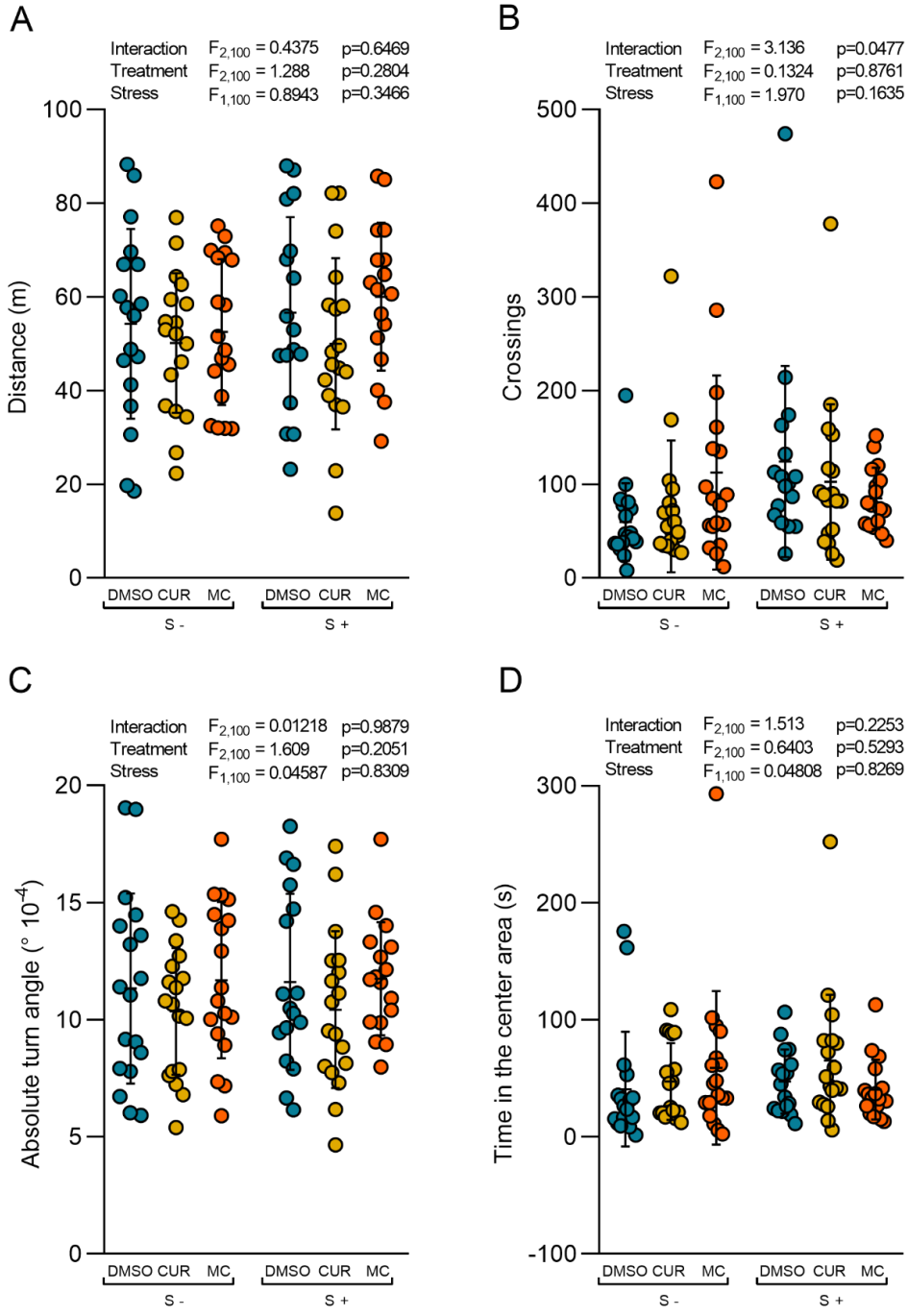


Fig. 8. Effects of CUR and MC (10 mg/kg) in zebrafish submitted to the UCS on the open tank test (day 17). (A) total distance traveled (B) the number of crossings, (C) absolute turn angle, (D) time in the center area. Data are expressed as mean ± S.D. Two-way ANOVA. n=17-18. DMSO (dimethyl sulfoxide); CUR (curcumin); MC (micronized curcumin).

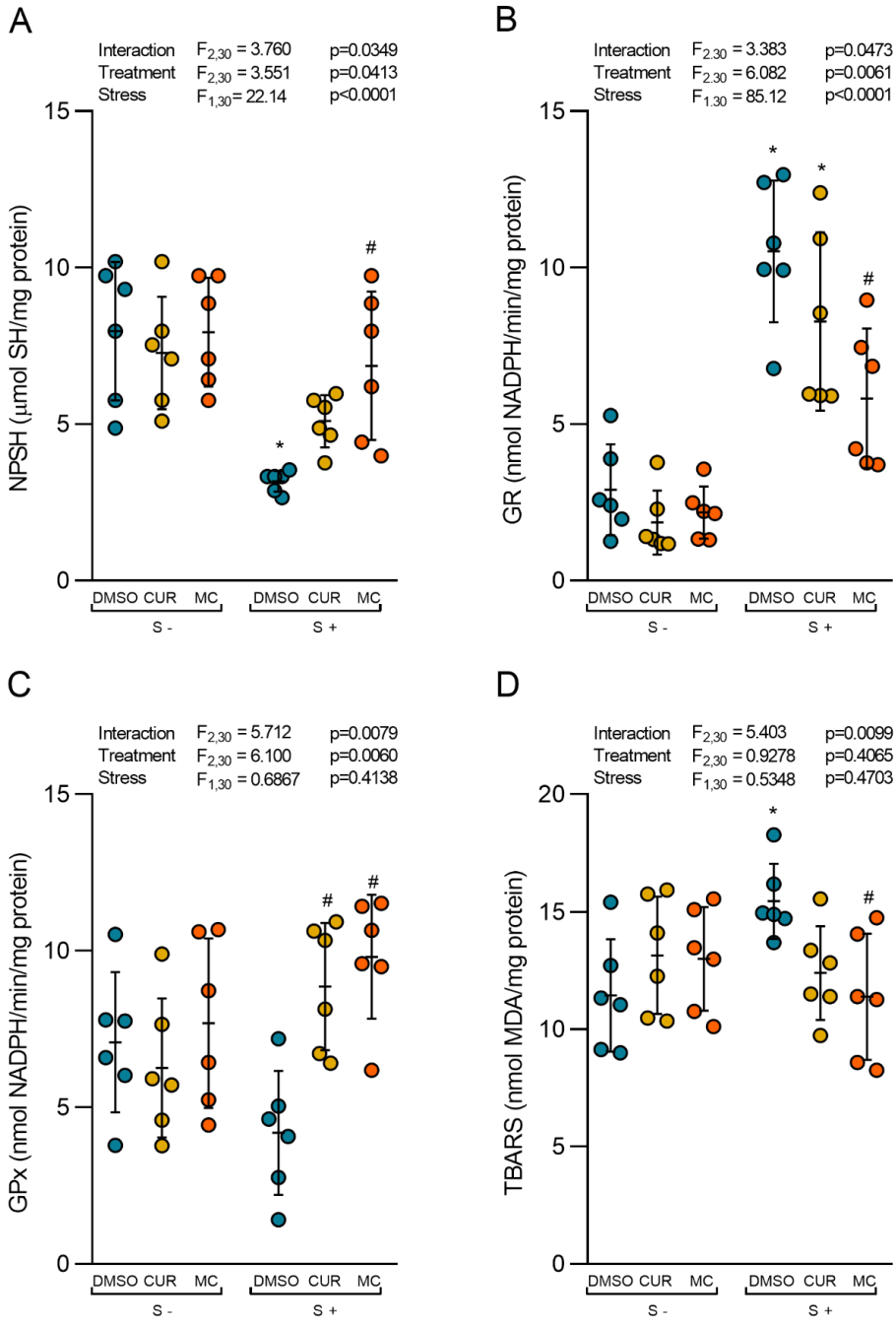


Fig. 9. Effects of CUR and MC (10 mg/kg) in zebrafish submitted to UCS on neurochemical parameters. (A) non-protein thiol levels, (B) glutathione reductase activity, (C) glutathione peroxidase activity, (D) lipid peroxidation levels. Data are expressed as mean ± S.D. Two-way ANOVA/Tukey. n=6. *p<0.05 x 1% DMSO non-stressed. #p<0.05 x 1% DMSO stressed. DMSO (dimethyl sulfoxide); CUR (curcumin); MC (micronized curcumin).

After the behavioral tests, we evaluated the effects of CUR and MC on neurochemical parameters in zebrafish submitted to UCS. Two-way ANOVA revealed an interaction between both factors for NPSH levels, GPx and GR enzyme activity, and TBARS levels. The post hoc analysis showed that UCS decreased the NPSH levels (a measure that reflects the levels of GSH) (Fig. 9A), increased the GR enzyme activity (Fig. 9B), and increased lipid peroxidation (measured by TBARS levels) (Fig. 9D). These results indicate an oxidative status disturbance UCS-induced and consequently oxidative damage in the zebrafish brain. Despite the two-way ANOVA revealed an interaction between both factors on the GPx enzyme activity, no significant effects were observed in post hoc analysis between the control non-stressed and stressed group. MC blocked the effects of UCS, normalizing GR activity, and increasing NPSH levels and GPx activity, consequently decreasing the lipid peroxidation. CUR was able to increase GPx enzyme activity, although it was unable to block the effects of the stress on lipid peroxidation, NPSH levels, and GR activity.

Some organs, like the brain, are more vulnerable to the detrimental effects of ROS because it has a high metabolic rate and lower antioxidant levels (Maes et al., 2011; Mandelker, 2008). Stress adaptation or allostasis increases cerebral energy demand, reflecting the increased mitochondrial activity within the brain, which is related to high oxygen consumption and greater ROS production (Avery, 2011; Harwell, 2007; Picard et al., 2018). Moreover, the increased production of ROS has been linked to hyperactivation of the HPA axis with a consequent increase in cortisol secretion by producing damage to hippocampal neurons, which maintain the homeostasis of the HPA axis by negative feedback mechanisms (Bhatia et al., 2011; KVETŇANSKÝ et al., 1995; Maes et al., 2011; SAPOLSKY et al., 1986). The overproduction of ROS and decrease in antioxidant defenses consequently cause an oxidative stress status that alters neuronal homeostasis and favors the occurrence of oxidative lesions in proteins, lipids, and nucleic acids, which may result in cell death (Avery, 2011; Harwell, 2007; Valko et al., 2007). The reduced GSH is one of the main antioxidant components involved in the removal of ROS and maintenance of oxidative status. GPx reduces H_2O_2 through the GSH oxidation to oxidized glutathione in

dimerized form (GSSG). GSSG is then recycled by the enzyme GR through the NADPH oxidation reaction to oxidized NADP. In addition, GSH also can undergo oxidation and form disulfides of the GSSR type with the cysteine thiol present in proteins. The sulfhydryl group present in the cysteine thiol is the active site and is responsible for its protective functions against oxidative stress. Therefore, its oxidation leads to the formation of GSH disulfides and inactivation of its antioxidant capacity, leaving the organism more susceptible to suffer oxidative damage (Dasuri et al., 2013; Gandhi and Abramov, 2012).

Our results suggest that unpredictable chronic stress decreased the antioxidant defenses by depleting cerebral GSH, making the zebrafish brain more susceptible to oxidative damage such as lipid peroxidation. On the other hand, UCS increased the activity of GR as a reflex of the organism in face of the decrease in GSH. Indeed, studies have shown the UCS increases body cortisol levels, at the same time that increase ROS production, decreases the antioxidant mechanisms (NPSH level and antioxidant enzyme activity superoxide dismutase (SOD)), and consequently increases lipid peroxidation (TBARS levels) in the zebrafish brain (Marcon et al., 2019, 2018b, 2018a; Mocelin et al., 2019). Interestingly, we also demonstrated for the first time that MC has a better protective effect than CUR against oxidative stress, blocking the stress-induced neurochemical effects in the zebrafish brain. We suggest that MC prevented oxidative stress by increasing antioxidant defenses (GSH level and GPx enzyme activity).

4. Conclusion

In this study, we have shown micronized curcumin prevented the effects of chronic stress on neurochemical markers, despite the absence of effects on behavioral parameters. Considering the heterogeneous and complex effects caused by chronic stress, one possibility is that curcumin would be exerting neuroprotective effects as an antioxidant, but not being able to modulate other systems involved in stress-induced behavioral changes. However, there is a clear superiority of the micronized

preparation over the conventional against stress-induced oxidative stress. Finally, the micronization with the SEDS technique altered the crystalline structure of the compound and its melting point, which led to significant improvements in both *in vivo* and *in vitro* tests. Thus, the micronization may increase bioavailability and potentiate the therapeutic effect of drugs, making the technology of supercritical fluid micronization promising to the pharmaceutical industry.

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Supplementary Material

Curcumin micronization by supercritical fluid: in vitro and in vivo biological relevance

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Table S1. Schedule and stressors of the unpredictable chronic stress protocol.

	Day/Time	1	2	3	4	5	6	7
STRESS	Morning	**10:30 AM** Tank change (3 times / 10 min each)	**08:00 AM** Overcrowding (9 animals in 200 mL beaker / 50 min)	**08:30 AM** Low housing tank water level until dorse exposure (2 min)	**09:30 AM** Heating tank water (33 ºC / 30 min)	**08:30 AM** Cooling tank water (23 ºC / 30 min)	**09:15 AM** Chasing with a net (8 min)	**8:00 AM** Tank change (3 times / 10 min each)
	Afternoon	**03:00 PM** Chasing with a net (8 min)	**04:30 PM** Low housing tank water level until dorse exposure (2 min)	**04:00 PM** Cooling tank water (23 ºC / 30 min)	**03:30 PM** Overcrowding (9 animals in 200 mL beaker / 50 min)	**04:00 PM** Tank change (3 times / 10 min each)	**04:30 PM** Low housing tank water level until dorse exposure (2 min)	**03:30 PM** Heating tank water (33 ºC / 30 min)

	Day/Time	8	9	10	11	12	13	14
STRESS + TREATMENT	Morning	**09:15 AM** Chasing with a net (8 min)	**08:00 AM** Tank change (3 times / 10 min each)	**09:15 AM** Cooling tank water (23 ºC / 30 min)	**08:30 AM** Overcrowding (9 animals in 200 mL beaker / 50 min)	**09:15 AM** Low housing tank water level until dorse exposure (2 min)	**08:15 AM** Heating tank water (33 ºC / 30 min)	**10:00 AM** Overcrowding (9 animals in 200 mL beaker / 50 min)
	Afternoon	**04:00 PM** Overcrowding (9 animals in 200 mL beaker / 50 min)	**03:00 PM** Chasing with a net (8 min)	**05:00 PM** Heating tank water (33 ºC / 30 min)	**03:30 PM** Chasing with a net (8 min)	**04:30 PM** Cooling tank water (23 ºC / 30 min)	**04:45 PM** Low housing tank water level until dorse exposure (2 min)	**04:00 PM** Tank change (3 times / 10 min each)

	Day/Time	15	16	17
STRESS + TREATMENT + TESTS	Morning	**08:00 AM** Social interaction test	**08:00 AM** Novel tank test	**08:00 AM** Open tank test and euthanasia
	Afternoon	**04:00 PM** Heating tank water (33 ºC / 30 min)	**04:30 PM** Cooling tank water (23 ºC / 30 min)	-

3.2. CAPÍTULO II: Non-micronized and micronized curcumin do not prevent the behavioral and neurochemical effects induced by acute stress in zebrafish.

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Non-micronized and micronized curcumin do not prevent the behavioral and neurochemical effects induced by acute stress in zebrafish

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ABSTRACT

Curcumin, a polyphenol extracted from the rhizome of *Curcuma longa* L. (Zingiberaceae), presents neuroprotective properties and can modulate neuronal pathways related to mental disorders. However, curcumin has low bioavailability, which can compromise its use. The micronization process can reduce mean particle diameter and improve this compound's bioavailability and therapeutic potential. In this study, we compared the behavioral (open tank test, OTT) and neurochemical (thiobarbituric acid reactive substances (TBARS) and non-protein thiols (NPSH) levels) effects of non-micronized curcumin (CUR, 10 mg/kg, i.p.) and micronized curcumin (MC, 10 mg/kg, i.p.) in adult zebrafish subjected to a 90-minute acute restraint stress (ARS) protocol. ARS increased the time spent in the central area and the number of crossings and decreased the immobility time of the animals in the OTT. These results suggest an increase in locomotor activity and a decrease in thigmotaxis behavior. Furthermore, ARS also induced oxidative damage by increasing TBARS and decreasing NPSH levels. ARS-induced behavioral and biochemical effects were not blocked by any curcumin preparation. Therefore, we conclude that curcumin does not have acute anti-stress effects in zebrafish.

Keywords: acute restraint stress, oxidative damage, curcumin, open tank test, zebrafish.

Introduction

The term stress refers to the set of responses triggered by the body in a situation assumed as threatening, which occurs due to a stressor. These responses are triggered by activation of the autonomic nervous system and hypothalamic-pituitary-adrenocortical (HPA) axis, which results in the release of catecholamines and glucocorticoids. It aims to adapt the system to a given demand, returning to the basal level of internal balance known as homeostasis. The persistence of these responses, even when the stress stimulus ends, indicates a failed adaptation, which can affect an individual's health status and result in mental disorders such as anxiety and depression [1–5]. Stress can lead to the depletion of the body's adaptive responses through complex neurobiological changes involving oxidative stress, neuroinflammation, neurotransmitter dysfunction, and excitotoxicity that can ultimately affect neurocircuits that regulate behavior, especially the ones relevant to motivation (anhedonia), avoidance, and alarm response (anxiety), which are related to several neuropsychiatric disorders [6]. Although effective treatments for these conditions are available, a significant number of patients do not adequately respond to the therapy, further contributing to the global burden of these mental diseases. Therefore, it is essential to study innovative treatments that modulate targets related to these psychopathologies [6,7].

Zebrafish stress response is mediated by the hypothalamic-pituitary-interrenal (HPI) axis that is functionally and structurally homologous to the mammalian HPA axis [8]. In response to an acute stressor (for example, restraint, alarm pheromone, net chasing), zebrafish exhibit a complex behavioral and physiological repertoire including anxiety, locomotor disturbance, increase in thigmotaxis behavior, cognitive impairment, hypercortisolemia, and oxidative status imbalance [9–21]. These effects are blocked by antidepressants/anxiolytics [9,16], antipsychotics [17], and other compounds [18,19,21].

Curcumin, a compound extracted from the roots of the ground turmeric (*Curcuma longa* L. Zingiberaceae), presented antioxidant, anti-inflammatory,

neuroprotective, immunomodulatory, anxiolytic, and antidepressant effects in several pre-clinical and clinical studies [7,22–26]. However, curcumin has low bioavailability, poor absorption, rapid metabolism, and quick systemic elimination, which compromise its therapeutic use for neuropsychiatric disorders [27,28]. Supercritical fluid micronization technology (SEDS) is an approach that has been used to modify material properties by reducing particle size, increasing dissolution rate and solubility, as well as modifying the crystal structure of the compound when compared to non-micronized compounds [29,30]. These changes can potentially increase bioavailability. Therefore, this study aimed to compare the effects of non-micronized curcumin and micronized curcumin on behavioral and biochemical parameters in adult zebrafish submitted to acute restraint stress.

Materials and methods

Drugs

Curcumin was obtained from Sigma-Aldrich® (CAS 458-37-7) (St. Louis, MO, USA). Curcumin micronization was carried out at the Laboratory of Thermodynamics and Supercritical Technology (LATESC) of the Department of Chemical and Food Engineering (EQA) at Universidade Federal de Santa Catarina (UFSC), with solution enhanced dispersion by supercritical fluids (SEDS) as previously described [31]. Both curcumin preparations were dissolved in 1% DMSO (dimethyl sulfoxide anhydrous) obtained from Sigma-Aldrich® (CAS 67-68-5) and diluted in injection water (Samtec Biotecnologia®, SP, Brazil) acquired from a commercial supplier. Reagents used for biochemical assays were obtained from Sigma Aldrich (St. Louis, MO, USA), 5,5′-dithiobis (2-nitrobenzoic acid) (CAS Number 69-78-3), thiobarbituric acid (CAS Number: 504-17-6), and trichloroacetic acid (CAS Number: 76-03-9). Absolute ethanol (CAS Number: 64-17-5) was obtained from Merck KGaA (Darmstadt, Germany).

Animals

All procedures were approved by the institutional animal welfare and ethical review committee at the Universidade Federal do Rio Grande do Sul (UFRGS) (approval #35279/2018). The animal experiments are reported in compliance with the ARRIVE guidelines 2.0 [32]. Experiments were performed at the Laboratory of Psychopharmacology and Behavior (LAPCOM) of Departamento de Farmacologia, UFRGS, using 144 6-month old male and female (50:50 ratio) short-fin wild-type zebrafish, weighing 300 to 400 mg. Adult animals were obtained from the colony established in the Biochemistry Department at UFRGS and maintained for at least 15 days before tests in our animal facility (Altamar, SP, Brazil) in 16-L home tanks (40 x 20 x 24 cm) with non-chlorinated water kept under constant mechanical, biological, and chemical filtration at a maximum density of two animals per liter. Tank water fulfilled the controlled conditions required for the species (26 ± 2 °C; pH 7.0 ± 0.3; dissolved oxygen at 7.0 ± 0.4 mg/L; total ammonia at <0.01 mg/L; total hardness at 5.8 mg/L; alkalinity at 22 mg/L $CaCO_3$; and conductivity of 1500–1600 µS/cm). The animals were maintained in a light/dark cycle of 14/10 hours and food was provided twice a day (commercial flake food (Poytara®, Brazil) plus the brine shrimp *Artemia salina*). After the tests, animals were euthanized by hypothermic shock according to the AVMA Guidelines for the Euthanasia of Animals [33]. Briefly, animals were exposed to chilled water at a temperature between 2 and 4 °C for at least 2 min after loss of orientation and cessation of opercular movements, followed by decapitation as a second step to ensure death.

Drug administration

Intraperitoneal (i.p.) injections were applied using a Hamilton Microliter™ Syringe (701N 10 µL SYR 26s/2"/2) x Epidural catheter 0.45 x 0.85 mm (Perifix®-Katheter, Braun, Germany) x Gingival Needle 30G/0.3 x 21 mm (GN injecta, SP, Brazil). The animal weight was checked 24 hours before the treatment and an average

between the weights of each tank was used to calculate the injection volume (1 µL/100 mg of animal weight). The animals were anesthetized by immersion in a solution of tricaine (300 mg/L, CAS number 886-86-2) until postural loss and reduction of respiratory rate. The anesthetized fish were gently placed in a sponge soaked in water placed inside a petri dish, with the abdomen facing up and the fish head positioned on the sponge hinge. The needle was inserted parallel to the spine in the abdomen midline posterior to the pectoral fins. This procedure was conducted in approximately 10 seconds. The behavioral tests took place 90 minutes after the injection.

Experimental design

The experimental design is presented in Fig. 1. Initially, animals were treated with 1% DMSO, 10 mg/kg CUR, or 10 mg/kg MC (n= 24). These doses were based in our previous study [31]. After the treatment, the experimental groups were subdivided into control (non-stressed group, S-) or acute restraint stress (ARS) (stressed group, S+). The stress protocol was conducted as described previously [10,12,15,20,21]. Fish in the stressed group were gently placed into 1.8-mL cryogenic tubes (Corning®) with openings at both ends (to allow adequate water circulation) in a 16-L tank for 90 min. Non-stressed groups were transferred to an identical 16-L tank for 90 min. After the acute stress protocol, the animals were individually transferred to the OTT, and behavioral parameters were quantified. Immediately after the OTT, fish were euthanized, and the brain was dissected and homogenized for the biochemical assays. The animals were allocated to the experimental groups following block randomization procedures to counterbalance the sex, the two different home tanks, and the test arenas between the groups. Animal behavior was video recorded and analyzed with the ANY-Maze tracking software (Stoelting Co., Wood Dale, IL, USA) by researchers blinded to the experimental groups. All tests were performed between 08:00 and 12:00 a.m. The sex of the animals was confirmed after euthanasia by dissecting and analyzing the gonads.

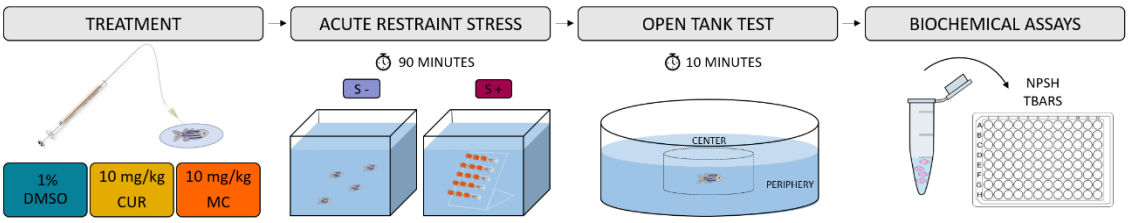

Fig 1. Experimental design. Treatments (1% DMSO, 10 mg/kg CUR, or 10 mg/kg MC) were injected intraperitoneally. Afterwards, zebrafish were subjected to acute restraint stress for 90 minutes. Zebrafish from the control group remained in an identical tank and were not submitted to stress. Subsequently, the animals were tested in the open tank test. Immediately after the behavioral test, each animal was euthanized and the brain dissected to performed the biochemical assays. DMSO (dimethyl sulfoxide), CUR (curcumin), MC (micronized curcumin), NPSH (non-protein thiols) and TBARS (thiobarbituric acid reactive substances).

Open tank test (OTT)

The OTT was conducted as described previously [10,31,34]. Animals were individually placed in the center of a circular arena made of opaque white plastic (24 cm diameter, 8 cm walls, 2 cm water level) and recorded for 10 min. The apparatus was virtually divided into two areas for video analysis: the central (12 cm in diameter) and the peripheral area. Videos were recorded from the top. The following parameters were quantified: total distance traveled (m), number of crossings (transitions between the areas of the tank), absolute turn angle (°), immobility time, time spent, and entries in the center area of the tank.

Neurochemical assays

For each independent sample, four brains were pooled (n=6) and homogenized in 600 µL of phosphate-buffered saline (PBS, pH 7.4, Sigma-Aldrich) and centrifuged at 10,000 g at 4 °C in a cooling centrifuge; the supernatants were collected and kept in microtubes on ice until the assays were performed. The detailed protocol for preparing brain tissue samples is available at protocols.io [35]. The protein content was quantified according to the Coomassie blue method using bovine serum albumin (Sigma-Aldrich)

as a standard [36]. The detailed protocol for protein quantification is available at protocols.io [37].

Non-protein thiols (NPSH)

The content of NPSH in the samples was determined by mixing equal volumes of the brain tissue preparation (50 µg of proteins) and trichloroacetic acid (TCA, 6%), centrifuging the mix (10,000 g, 10 min at 4 °C), the supernatants were added to TFK (1 M) and DTNB (10 mM) and the absorbance was measured at 412 nm after 1 h. The detailed protocol is available at protocols.io [38].

Thiobarbituric acid reactive substances (TBARS)

The lipid peroxidation was evaluated by quantifying the production of TBARS. Samples (50 µg of proteins) were mixed with TBA (0.5%) and TCA (20%) (150 µL). The mixture was heated at 100 °C for 30 min. The absorbance of the samples was determined at 532 nm in a microplate reader. MDA (2 mM) was used as the standard. The detailed protocol is available at protocols.io [39].

Statistical analysis

We calculated the sample size to detect an effect size of 0.35 for the interaction between stress and treatment with a power of 0.9 and an alpha of 0.05 using G*Power 3.1.9.7 for Windows. The total distance traveled was defined as the primary outcome. The total sample size was 144, equivalent to n = 24 animals per experimental group.

The normality and homogeneity of variances were confirmed for all data sets using D'Agostino-Pearson and Levene tests, respectively. Results were analyzed by two-way ANOVA. The outliers were defined using the ROUT statistical test and were removed from the analyses. This resulted in 6 outliers being removed from the OTT test (1 animal from CUR S-, 2 animals from DMSO S+ and 3 animals from MC S+

groups). Moreover, one animal showed 0 distance in OTT from the MC S+ group and was removed from the test. The tank and sex effects were tested in all comparisons and no effect was observed, so the data were pooled.

Data are expressed as mean ± standard deviations of the mean (S.D.). The level of significance was set at $p<0.05$. Data were analyzed using IBM SPSS Statistics version 27 for Windows and the graphs were plotted using GraphPad Prism version 8.0.1 for Windows.

Results

Behavioral parameters

The effects of CUR and MC on behavioral parameters in zebrafish submitted to ARS in the open tank test (OTT) are presented in Fig 2. Two-way ANOVA revealed that acute stress increased the number of crossings (Fig. 2B) and decreased the time spent immobile (Fig. 2D). Also, ARS increased the time spent in the center area (Fig. 2E), indicating a decrease in the thigmotaxic behavior. Both CUR and MC did not prevent the effects of acute stress on these behavioral parameters. There was no statistical difference in the parameters of total distance traveled, absolute turn angle, and center entries.

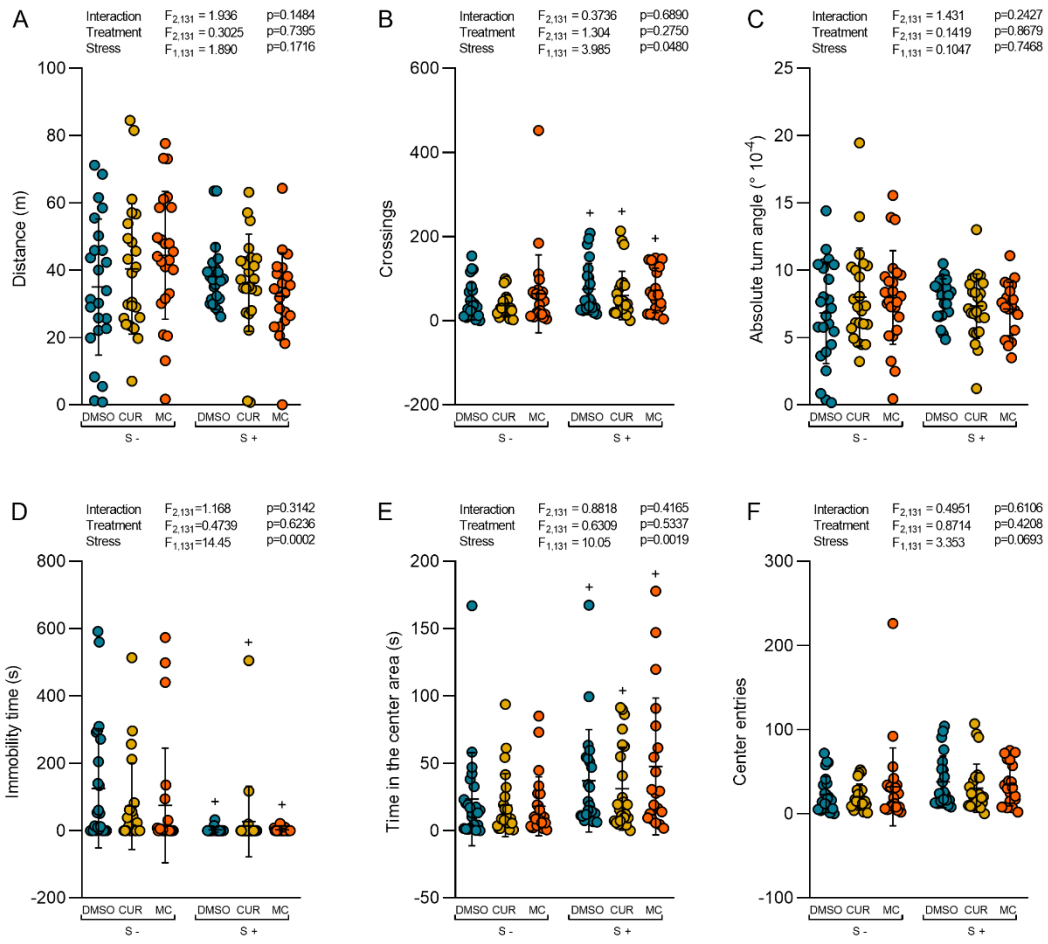

Fig 2. Effects of CUR and MC (10 mg/kg) on behavioral parameters in zebrafish submitted to the acute restraint stress. (A) total distance traveled, (B) number of crossings, (C) absolute turn angle, (D) immobility time, (E) time in the center area, and (F) center entries. Data are expressed as mean ± S.D. Two-way ANOVA. n=20-24. $^{+}p<0.05$ stress effect. DMSO (dimethyl sulfoxide), CUR (curcumin), MC (micronized curcumin).

Neurochemical parameters

The effects of CUR and MC on oxidative status parameters in zebrafish brains submitted to ARS are presented in Fig 3. Two-way ANOVA revealed a main effect of stress on NPSH (Fig. 3A) and TBARS (Fig. 3B) levels, decreasing and increasing,

respectively. These results indicate ARS provoked oxidative stress in the zebrafish brain. Both CUR and MC were not able to prevent these effects.

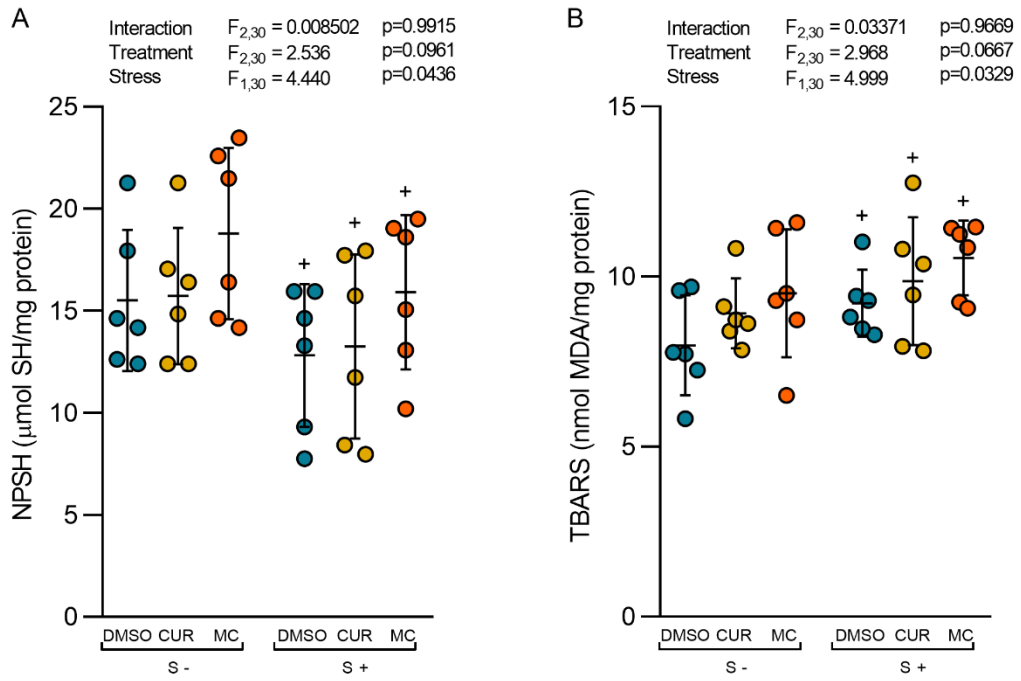

Fig 3. Effects of CUR and MC (10 mg/kg) on neurochemical parameters in zebrafish submitted to the acute restraint stress. (A) NPSH and (B) TBARS. Data are expressed as mean ± S.D. Two-way ANOVA. n=6. $^{+}p<0.05$ stress effect. DMSO (dimethyl sulfoxide), CUR (curcumin), MC (micronized curcumin), NPSH (non-protein thiols), TBARS (thiobarbituric acid reactive substances).

Discussion

In this study, ARS increased locomotion and decreased thigmotaxis behavior in the open tank test, while induced oxidative damage in the zebrafish brain. Both curcumin preparations were not able to prevent the effects of ARS on the behavioral and neurochemical parameters tested.

The acute restraint stress model merges both emotional and physical aspects of stress and is widely used to study the impact of stress on disease processes and in stress-associated pathological conditions [40]. In response to an acute stressor, zebrafish

may display fear/anxiety-like behaviors, such as altered locomotion (e.g., increased distance and average speed spent in the tank) as well as memory deficits and increase of cortisol levels [10,11,13,16,20,21].

Traditionally, an increase in the time and exploration of aversive zones (bolder behavior) in open field tests are usually regarded as anxiolytic effects [41,42]. Acute restraint stress has been reported to alter locomotor activity and cause anxiety-like behaviors in zebrafish [10,20,21]. In this study, ARS increased locomotion and decreased thigmotaxis in the open tank test. These findings, therefore, could suggest that acute stress may momentarily relieve anxiety in zebrafish, which appears counterintuitive but is not unprecedented in the literature [11]. The same was observed in zebrafish submitted to the acute restraint stress (15 minutes), which showed an increase in the number of the crossings, and spent significantly less time in the external area (periphery) than non-stressed controls in the OTT [11]. Also, acute stress has been previously shown to affect behavioral patterns in rodents in a nonconventional manner as well. For instance, in a study in rodents, acute immobilization (120 minutes) produced anxiety-like behavior in the elevated plus-maze, which is reflected by hyperactivity in the open field test [40]. Immediate exposure to stress has been reported to induce anxiogenic behavior that may result in an excitable and irritable state leading to impaired performance [40,43]. Furthermore, acute stress paradigms including restraint stress can cause a reduction in anxiety-like behaviors such as risk assessment behaviors (head poking and stretch attempts) and at the same time cause increases in the frequency of direct entries in the inner/anxiogenic zone of a novel environment such as the open field [11]. The reason underlying this phenomenon is unclear. One possibility is that acute stress may momentarily alter cognitive function, which may, in turn, affect the selection of appropriate risk assessment behaviors and/or coping strategies [11]. Moreover, the distance was not changed by ARS, so this behavior is presumably not driven by general behavioral hyperactivity but rather by a voluntary decision not to visit the periphery zone as non-stressed controls [11]. These findings suggest that stressed zebrafish seem to adopt a coping style that is less suited for successful escape and therefore might not be adaptive (e.g., increases the risk for

predator attacks), thus supporting the view that acute stress may impair cognitive abilities. It is also possible that stress could act as a "disorienting" factor causing zebrafish to lose the ability to discriminate between the different parts of the open field [11].

The stimulation of intracellular pathways induced by stress also leads to the free radical generation from intracellular reactive oxygen species, such as superoxide, hydrogen peroxide, and hydroxyl radicals resulting in an imbalance of antioxidant status, disturbances in homeostasis, and oxidative stress [40,44]. In our study, ARS lowered GSH (NPSH) levels, making the zebrafish brain more susceptible to oxidative damage such as increased lipid peroxidation (TBARS). Numerous reports have revealed that restraint stress can affect central nervous system functions by producing neurochemical and hormonal abnormalities associated with oxidative stress in zebrafish [10,12,21]. Also, many studies have shown that restraint stress increases lipid peroxidation and increases or decreases antioxidant enzyme activities in different brain regions of rodents depending on the severity and duration of immobilization stress protocol from inducing numerous cellular cascades that lead to increased reactive oxygen species production [40]. In our study, both curcumin preparations were not able to prevent the ARS-induced effects on behavioral and biochemical parameters in zebrafish.

There is controversy in the literature about the anxiolytic effects of curcumin. In rodents subjected to acute immobile stress (120 minutes), both anxiety and hyperactivity were reversed by pre-treatment with curcumin (200 mg/kg/day for 7 days) in the open field test [40]. Also, the pre-treatment with curcumin (20 mg/kg) prevented the hyperlocomotion and anxiogenic state induced by restraint stress (6 hours) in mice [40,45]. However, in another study, curcumin (20 mg/kg) did not demonstrate any effects in the open field test, and no interaction of curcumin at the benzodiazepine site of the $GABA_A$ receptor was observed [46].

Curcumin has recently been classified as both a PAINS (pan-assay interference compounds) and an IMPS (invalid metabolic panaceas) candidate. Curcumin has shown promise in thousands of preclinical studies. However, over 100 clinical trials

have failed to find health benefits in humans against several diseases. No form of curcumin, or its closely related analogs, appears to possess the properties required for a good drug candidate (chemical stability, high water solubility, potent and selective target activity, high bioavailability, broad tissue distribution, stable metabolism, and low toxicity). The essential medicinal chemistry of curcumin provides evidence that curcumin is an unstable, reactive, and nonbioavailable compound. Moreover, the available evidence demonstrates that curcumin will ultimately degrade upon release into physiologic media, no matter the delivery mechanism. [47]. Acute curcumin does not seem to have an anti-stress effect on behavior parameters in zebrafish. In our previous study, both curcumin and micronized curcumin were unable to block the behavioral effects of chronic stress (14 days) in zebrafish, even after chronic treatment (7 days), and only antioxidant effects were observed, which were not sufficient to block its behavioral effects [31].

Conclusions

In this study, we have shown that ARS increased locomotion and decreased thigmotaxis in the open tank test and induced oxidative damage in the zebrafish brain. We infer that acute stress may impair cognitive abilities acting as a disorienting factor causing a reduction in anxiety-like behaviors such as risk assessment behaviors in zebrafish. Moreover, both curcumin preparations were not able to prevent the effects of ARS on behavioral and biochemical parameters in zebrafish. These results corroborate with previous data showing curcumin (for 7 days) did not seem to have a behavioral effect in zebrafish, even after the micronization process. We cannot rule out that curcumin might exert effects on stress-induced behavioral changes with a longer exposure time or in a dose range different from that used in this study.

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Author contributions

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Conceptualization, A.S. and A.P.; Methodology, A.S., R.B., M.G-L., A.M.L.; M.M., G.P.S.A., J.V.O., A.M.S., A.P.; Investigation, A.S., R.B., M.G-L., A.M.L.; M.M., G.P.S.A.; Formal Analysis, R.B., M.M., A.P. and A.P.H.; Resources, J.V.O., A.M.S., A.P.; Writing - Original Draft, A.S.; Writing - Review & Editing, A.S., R.B., M.G-L., M.M., A.M.L., G.P.S.A., A.P.H., J.V.O., A.M.S., A.P.; Supervision, A.P.; Funding Acquisition, A.P.

Competing interests

The authors declare no competing interests.

Data availability

The datasets generated in the current study are available from the corresponding author on reasonable request.

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3.3. CAPÍTULO III: Micronized curcumin causes hyperlocomotion in zebrafish larvae.

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Micronized curcumin causes hyperlocomotion in zebrafish larvae

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ABSTRACT

Zebrafish larvae have been widely used in neuroscience and drug research and development. In the larval stage, zebrafish present a broad behavioral repertoire and physiological responses similar to adults. Curcumin (CUR), a major component of *Curcuma longa* L. (Zingiberaceae), has demonstrated the ability to modulate several neurobiological processes relevant to mental disorders in animal models. However, the low bioavailability of this compound can compromise its *in vivo* biological potential. Interestingly, it has been shown that micronization can increase the biological effects of several compounds. Thus, in this study, we compared the effects of acute exposure for 30 minutes to the following solutions: water (control), 0.1% DMSO (vehicle), 1 μM CUR, or 1 μM micronized curcumin (MC) in zebrafish larvae 7 days post-fertilization (dpf). We analyzed locomotor activity (open tank test), anxiety (light/dark test), and avoidance behavior (aversive stimulus test). Moreover, we evaluated parameters of oxidative status (thiobarbituric acid reactive substances and non-protein thiols levels). MC increased the total distance traveled and absolute turn angle in the open tank test. There were no significant differences in the other behavioral or neurochemical outcomes. The increase in locomotion induced by MC may be associated with a stimulant effect on the central nervous system, which was evidenced by the micronization process.

Keywords: curcumin, behavior, antioxidant, development, larvae, locomotion.

INTRODUCTION

Zebrafish have been used as a model organism for biomedical research in drug discovery, developmental biology, and genetics as it presents high mammalian homology [1]. Zebrafish present rapid development and a relatively long lifespan, being used to investigate the neurobehavioral foundations of various neurological and psychiatric conditions such as epilepsy, Alzheimer's and Parkinson's disease, schizophrenia, and stress- and drug-related disorders [1–9].

The low cost of rearing and maintenance and the numerous offspring are some of the attractive features of this organism when compared to rodents. This organism has proven to be very versatile in research, being used at different stages of development. Zebrafish larvae have a rich behavioral repertoire and respond to pharmacological and non-pharmacological interventions similarly to rodents [1, 8]. Studies have shown that zebrafish larvae respond to different classes of psychotropic drugs including ethanol, hallucinogens, and nicotine [10–15]. All these aspects support the use of zebrafish larvae in high-throughput screening for research and development of new drugs as well as for repurposing [2, 16].

Curcumin is the major active component extracted from the roots of turmeric *Curcuma longa* L. (Zingiberaceae). In larval and adult zebrafish, it has shown antioxidant [17–20], anti-seizure [5, 21], anti-inflammatory [20] and cognitive enhancing [22] activities. However, curcumin has low bioavailability *in vivo*, compromising its use [23, 24]. Micronization, a process that uses a supercritical fluid to change physicochemical aspects of substances, has been shown to improve the biological effects of substances such as N-acetylcysteine, resveratrol, and curcumin [5, 25–28]. In our previous studies, both curcumin and micronized curcumin (10 mg/kg, intraperitoneal) were unable to block the behavioral effects of acute restraint stress (90 minutes) [29] and unpredictable chronic stress (14 days) [28], while robust antioxidant effects were observed for micronized but not for its conventional preparation in adult zebrafish [28].

Larval stage zebrafish may occupy different ecological niches and face

different environmental demands (e.g., type of predators, social behavior, preference for certain environments) when compared to adult zebrafish and therefore the behavioral phenotypes can be distinct [30]. In addition, zebrafish larvae have a more permeable blood-brain barrier (BBB) than adults and increased absorption of small molecules in water as well [31]. Thus, considering the pharmacokinetic limitations of curcumin, tests in larvae may respond differently from those observed in adults, and introducing the compound in water can better evidence the effects of micronization on the bioavailability. Therefore, this study aimed to compare the effects of exposure to conventional and micronized curcumin on locomotor, anxiety, cognitive, and biochemical parameters in zebrafish larvae.

MATERIALS AND METHODS

Drugs

Curcumin was obtained from Sigma-Aldrich™ (CAS 458-37-7) (St. Louis, MO, USA). Its micronization was carried out at the Laboratory of Thermodynamics and Supercritical Technology (LATESC) of the Departmento de Engenharia Química e de Alimentos (EQA) at Universidade Federal de Santa Catarina (UFSC), with the enhanced dispersion solution by supercritical fluids (SEDS) according to SACHETT et al. (2021a). Both curcumin preparations were dissolved in 0.1% dimethyl sulfoxide anhydrous (DMSO) obtained from Sigma-Aldrich (CAS 67-68-5) and diluted in the system water. Reagents used for biochemical assays were obtained from Sigma Aldrich (St. Louis, MO, USA): bovine serum albumin (CAS Number 9048-46-8), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) (CAS Number 69-78-3), thiobarbituric acid (TBA) (CAS Number: 504-17-6), and trichloroacetic acid (TCA) (CAS Number: 76-03-9). Absolute ethanol (CAS Number: 64-17-5) was obtained from Merck KGaA (Darmstadt, Germany).

Animals

All procedures were approved by the institutional animal welfare and ethical review committee at the Universidade Federal do Rio Grande do Sul (UFRGS) (approval #35279/2018). The experiments are reported in compliance with the ARRIVE guidelines 2.0 [32]. The embryos used were obtained from mating wild-type zebrafish (*Danio rerio*) from the colony of the Departamento de Bioquímica at UFRGS. The animals were maintained in recirculating systems (Zebtec™, Tecniplast, Italy) with reverse osmosis filtered water equilibrated to reach the species standard parameters including temperature (28 ± 2 °C), pH (7 ± 0.5), conductivity and ammonia, nitrite, nitrate, and chloride levels. The total organic carbon concentration was 0.33 mg/L and the total alkalinity (as carbonate ion) was 0.030 mEq/L. The animals were kept with a light/dark cycle of 14/10 h. The system water used in the experiments was obtained from a reverse osmosis apparatus (18 MOhm/cm) and was reconstituted with marine salt (Crystal SeaTM, Marinemix, Baltimore, MD, USA) at 0.4 ppt. For the breeding, in a breeding tank, females and males (1:2) were separated overnight by a transparent barrier, which was removed after the lights went on the following morning. The fertilized eggs retained in the bottom of the fitted tank were collected 1 hour after fertilization and the viable embryos were sanitized. They were randomly relocated in 24-well plates (4 embryos per well with 2 mL of system water), and kept in a biochemical oxygen demand incubator (BOD), on the same shelf to ensure the same lighting and housing pattern. The temperature was constant (28 °C) with a light/dark period of 14/10 hours until the 7^{th} day post-fertilization (dpf). At 7 dpf, the larvae were exposed to treatments and submitted to behavioral or biochemical tests. All experiments were performed in duplicate to confirm the results. Since zebrafish larvae can absorb all the necessary nutrients through the yolk sac up to 7 dpf, it was not necessary to feed the animals during the experiment. Embryos were evaluated daily to monitor mortality and hatching rates. At the end of the experiments, the zebrafish larvae were euthanized by immersion in cold water (0 to 4 ºC) for 20

minutes until the cessation of the movements, to ensure death by hypoxia according to the AVMA Guidelines for the Euthanasia of Animals [33].

Experimental design

The experiments were performed according to Figure 1. Two different experimental sets were carried out for the behavioral (open tank and light/dark (n = 16), and aversive stimulus tests (n = 4)) and another for the biochemical tests (levels of non-protein thiols and thiobarbituric acid reactive substances) (n = 3, pool = 15).

At 7 dpf, the animals were randomly allocated to the experimental groups, 4 larvae per well, in a 24-well plate (2 mL) for the behavioral tests and 15 larvae per well, in a 6-well plate (5 mL) for biochemical tests. The animals were allocated following block randomization procedures to counterbalance the housing plate (2 different plates). The larvae were exposed for 30 minutes to the following treatments: water (control), 0.1% DMSO (dilution vehicle), 1 μM curcumin (CUR), or 1 μM micronized curcumin (MC). Both curcumin preparations were diluted in 0.1% DMSO. All treatments were administered by immersion, being the substances dissolved in the system water. Concentrations were pre-established based on the literature [5]. All experimenters who analyzed the data were blind to the treatment groups.

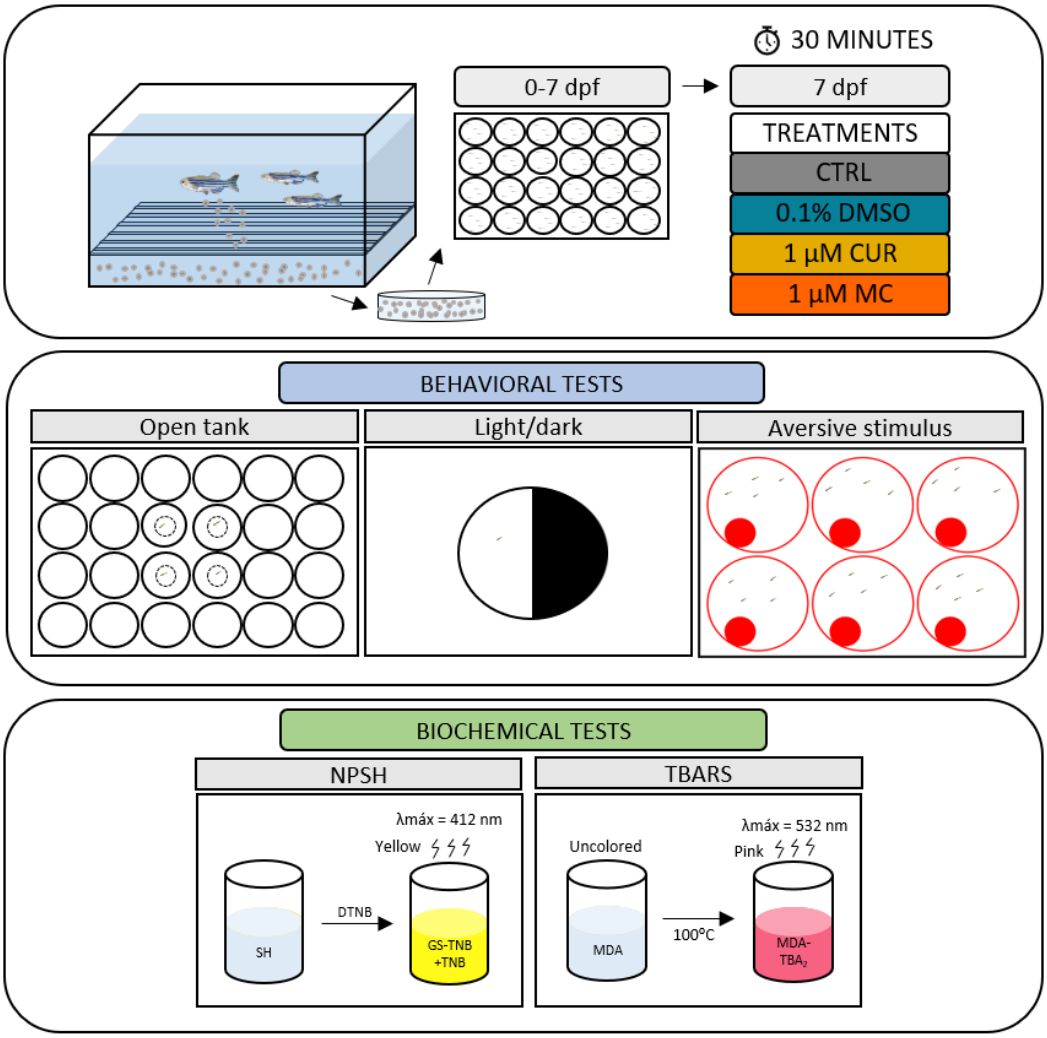

Figure 1. Experimental design. Two different sets of animals were used for the behavioral (set 1) and biochemical (set 2) tests. Initially, zebrafish larvae were randomly allocated to the experimental groups, 4 larvae per well, in a 24-well plate for the set 1 and 15 larvae per well, in a 6-well plate for set 2. At 7 dpf the larvae were exposed for 30 minutes to the following treatments: water (control), 0.1% DMSO (dilution vehicle), 1 µM CUR, or 1 µM MC. Subsequently, set 1 was tested in the open tank, light/dark (n = 16), and aversive stimulus tests (n = 4), and set 2 was assayed for NPSH and TBARS levels (n = 3, pool = 15). Dpf (days post-fertilization), CTRL (control), DMSO (dimethyl sulfoxide), CUR (curcumin), MC (micronized curcumin), NPSH (non-protein thiols) and TBARS (thiobarbituric acid reactive substances).

Open tank test (OTT)

To assess behavioral effects of treatments on locomotor and exploratory parameters the OTT was performed [2, 15, 34]. After 30 minutes of exposure to the treatments, the 7 dpf larvae were transferred individually to a 24-well plate filled with 2 mL of system water. The behavior was recorded from the top view and analyzed for 5 min, following 1 min of acclimation. The videos were analyzed with the ANYmaze® software (Stoelting Co., USA). The apparatus was virtually divided into two areas for video analysis: the central area of 8 mm in diameter and the periphery of 8 mm. The following parameters were quantified: total distance (m), crossings (transitions between the areas of the well), absolute turn angle (º), immobility time (s), and center time (time spent in the center area of the well) (s).

Light/dark test (LDT)

After the OTT, the LDT was performed to assess anxiety parameters [30]. The larvae were individually placed on the light (white) side of the light/dark arena, which consists of a petri dish (60 x 15 mm) divided equally into two areas, light (white) and dark (black), filled with 15 mL of system water. The animals were recorded for 5 minutes, and the behavior was analyzed with BORIS® software. The time spent in the light area was measured.

Aversive stimulus test (AST)

After the LDT, the avoidance capacity and cognitive responses to a visual stimulus were evaluated using an aversive task [35, 36]. The larvae were placed in 6-well plates (4 larvae per well, n = 4, filled with 5 mL of system water. The plates were placed on an LCD screen and exposed to an aversive visual stimulus. The video presented a red-filled circle that oscillates between the two ends of the well for 5 minutes. A habituation phase was carried out for 2 minutes before the stimulus started.

The larvae were recorded and the time spent in the half without stimulation was evaluated. Animals that remained in the area with stimulation were considered to have cognitive and avoidance capacity dysfunction.

Biochemical parameters

To assess the oxidative status, 30 minutes after exposure to treatments, biochemical tests were performed. Each pool of 15 larvae was simultaneously euthanized in a Petri dish with ice water at 0-4 °C. Afterward, all larvae were placed in a microtube (remaining water was removed) and 350 µL of phosphate-buffered saline (PBS, pH 7.4, Sigma-Aldrich) was added, then homogenized 60x with a pistil and centrifuged at 10,000 g at 4 °C in a refrigerated centrifuge. The supernatants were collected and kept in microtubes on ice until the assays were performed. The protein content was quantified according to the coomassie blue method using bovine serum albumin as a standard [37]. The detailed protocol for protein quantification is available at protocols.io [38].

Non-protein thiols (NPSH)

The content of NPSH in the samples was determined by mixing equal volumes of the samples (50 µg of proteins) and TCA (6%). The mix was centrifuged (10,000 g, 10 min at 4 °C) and the supernatants were added to potassium phosphate buffer (TFK, 1 M) and DTNB (10 mM). After 1 h, the absorbance was measured at 412 nm. The detailed protocol is available at protocols.io [39].

Thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation was evaluated by quantifying the production of TBARS. Samples (50 µg of proteins) were mixed with TBA (0.5%) and TCA (20%). The mixture was heated at 100 °C for 30 min. The absorbance was measured at 532 nm in

a microplate reader. MDA (2 mM) was used as the standard. The detailed protocol is available at protocols.io [40].

Statistical analysis

We calculated the sample size to detect an effect size of 0.5 with a power of 0.9 and an alpha of 0.05 using G*Power 3.1.9.7 for Windows. The total distance traveled in OTT was defined as the primary outcome. The total sample size was 64, n = 16 animals per experimental group.

The normality and homogeneity of variances were confirmed for all data sets using D'Agostino-Pearson and Levene tests, respectively. Results were analyzed by one-way ANOVA followed by Tukey's *post hoc* test when appropriate. The outliers were defined using the ROUT statistical test using the total distance traveled as the primary outcome and were removed from the analyses. This resulted in 5 outliers being removed from the OTT test (1 animal from CTRL, DMSO, and MC and 2 animals from CUR groups). The house plate effect was tested in all comparisons and no effect was observed, so the data were pooled.

Data are expressed as mean ± standard deviations of the mean (S.D.). The level of significance was set at $p<0.05$. Data were analyzed using IBM SPSS Statistics version 27 for Windows and the graphs were plotted using GraphPad Prism version 8.0.1 for Windows.

RESULTS

Behavioral parameters

The effects of CUR and MC on behavioral parameters in larvae zebrafish submitted to OTT are presented in Figure 2. Acute MC exposure increased the distance ($F_{3,55} = 5.142$, $p=0.0033$) (Fig. 2A) and absolute turn angle ($F_{3,55} = 3.688$, $p=0.0378$) (Fig. 2C). One-way ANOVA revealed a significant effect on the

immobility time (Fig. 2D); however, no significant effects were observed in *post hoc* analysis. These results indicate an increase in locomotion and absolute turn angle in zebrafish larvae exposed to MC. There was no statistical difference of any intervention in the number of crossings and center time. Table 1 summarizes the one-way ANOVA analysis.

There was no statistical difference of any intervention in the time in the lit side in the LDT (Fig. 3), neither in percent of the animals in non-stimulus area in the AST (Fig. 4).

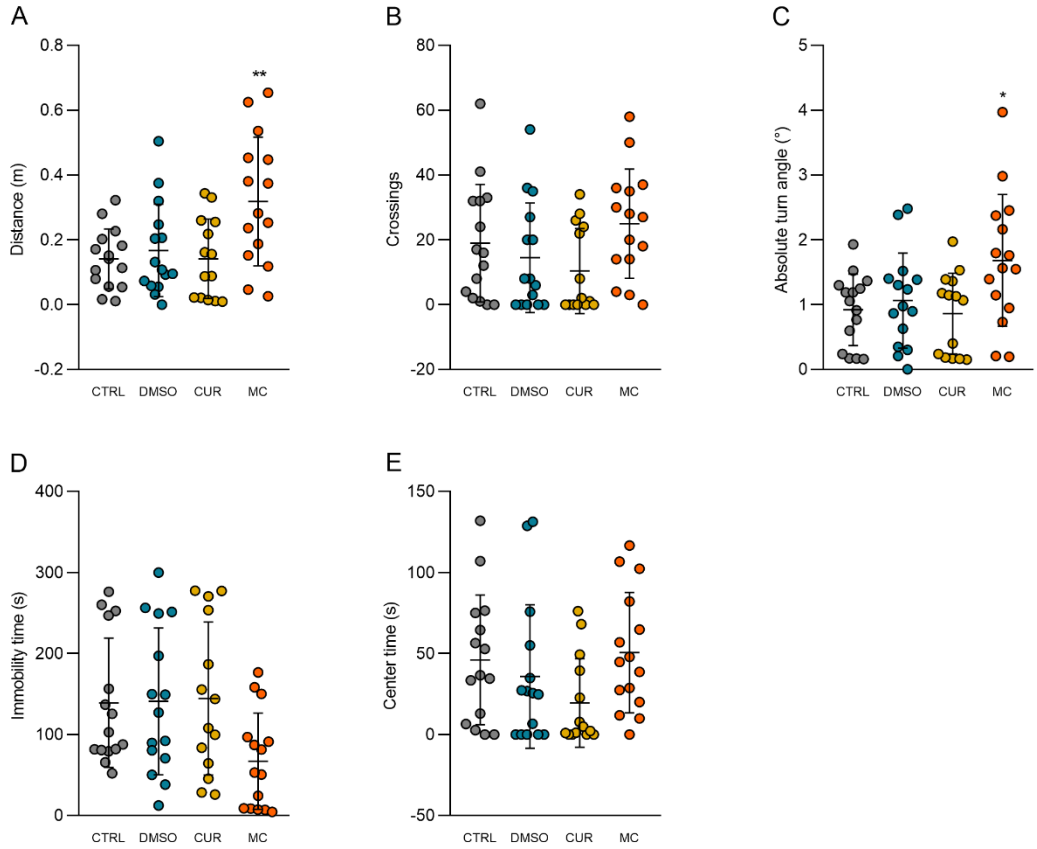

Figure 2. Effects of CUR and MC (1 µM) on behavioral parameters in the open tank test. (A) distance, (B) crossings, (C) absolute turn angle, (D) immobility time, and (E) center time. Data are expressed as mean ± S.D. One-way ANOVA/Tukey. n = 14-15. $^{*}p<0.05$ vs. CTRL. $^{**}p<0.005$ vs. CTRL. CTRL (control), DMSO (dimethyl sulfoxide), CUR (curcumin), MC (micronized curcumin).

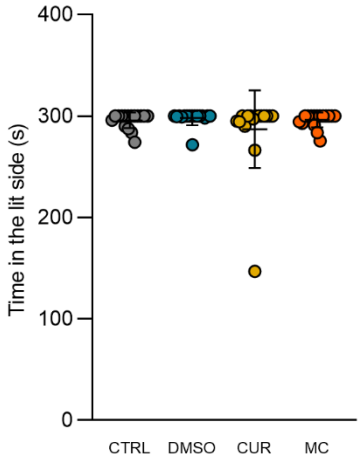

Figure 3. Effects of CUR and MC (1 µM) on behavioral parameters in the light/dark test. Data are expressed as mean ± S.D. One-way ANOVA. n = 16. CTRL (control), DMSO (dimethyl sulfoxide), CUR (curcumin), MC (micronized curcumin).

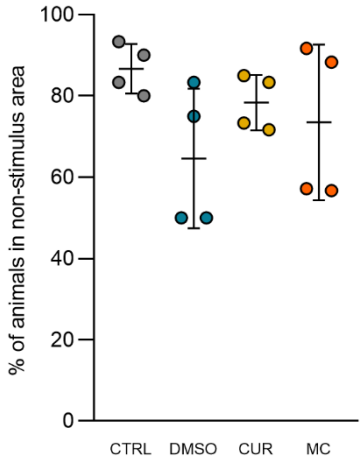

Figure 4. Effects of CUR and MC (1 µM) on behavioral parameters in the aversive test. Data are expressed as mean ± S.D. One-way ANOVA. n = 4. CTRL (control), DMSO (dimethyl sulfoxide), CUR (curcumin), MC (micronized curcumin).

Biochemical parameters

The effects of CUR and MC on oxidative status parameters in zebrafish larvae are presented in Figure 5. There was no statistical difference of any intervention in the non-protein thiols and thiobarbituric acid reactive substances levels.

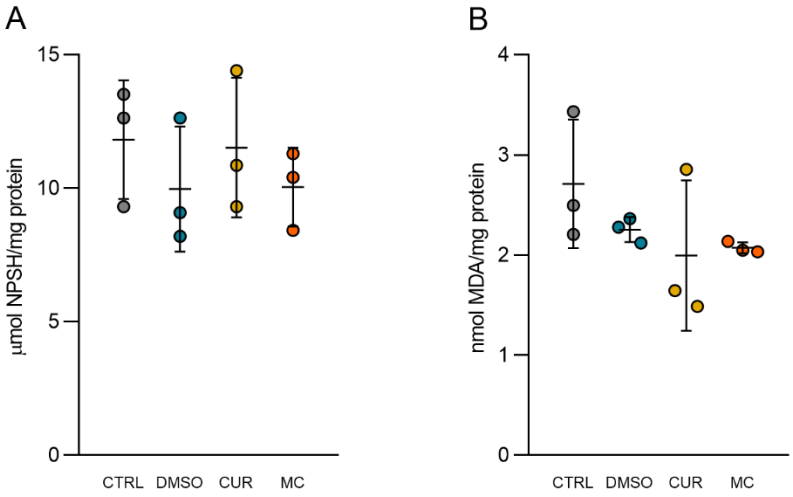

Figure 5. Effects of CUR and MC (1 µM) on biochemical parameters. (A) NPSH and (B) TBARS. Data are expressed as mean ± S.D. One-way ANOVA. n = 3. CTRL (control), DMSO (dimethyl sulfoxide), CUR (curcumin), MC (micronized curcumin), MDA (malondhyaldeide), NPSH (non-protein thiols), TBARS (thiobarbituric acid reactive substances).

Table 1. Summary of the one-way ANOVAs for the open tank, light/dark, aversive and biochemical tests.

Test	Dependent variable	F-value	DF	P-value
Open tank test	Distance	5.142	3,55	**<0.05**
	Crossings	2.109	3,55	>0.05
	Absolute turn angle	3.688	3,55	**<0.05**
	Immobility time	3.079	3,55	**<0.05**
	Center time	1.907	3,55	>0.05
Light/dark test	Time in the lit side	0.9696	3,60	>0.05

Aversive stimulus test	% of animals in the non-stimulus area	1.830	3,12	>0.05
Biochemical test	NPSH	1.250	3,8	>0.05
	TBARS	0.5782	3,8	>0.05

Significant effects ($p<0.05$) are given in bold font. DF (degrees of freedom), NPSH (non-protein thiols), TBARS (thiobarbituric acid reactive substances).

DISCUSSION

In this study, MC increased locomotion and absolute turn angle in the open tank test. However, there were no significant differences in the anxiety parameters in the light/dark or cognitive alterations in the aversive stimulus tests by any treatment. Also, there were no changes in the oxidative status of zebrafish larvae exposed to CUR or MC.

Essential for the survival of zebrafish, locomotion is a complex behavior controlled by the activity of various reticulospinal neurons of the brain stem along with descending vestibulospinal or neuromodulatory projections, whose activities depend on the integrity of brain function, nervous system development, and visual pathways [16, 41, 42]. The distance traveled and the pattern of movement of zebrafish larvae are relevant parameters to understand the neurobehavioral effects of the interventions [16]. The absolute turn angle is a measure of turning irrespective of its direction, which correlates with the motor pattern and is used to evaluate the motor coordination [43, 44]. In this study, MC increased the distance traveled and the absolute turn angle. Similar to our results, acute administration of neuroactive drugs that indirectly alter dopaminergic (DA) signaling as ethanol, d-amphetamine, and cocaine, increased locomotion in zebrafish larvae, and these effects were also observed in mammals [11, 12, 45]. Both the D_1 and D_2 selective agonists (SKF-38393 and quinpirole, respectively) increased locomotor activity, while the selective antagonists (SCH-23390 and haloperidol, D_1- and D_2- antagonists respectively)

decreased locomotion [12]. Also, ethanol and quinpirole increased absolute turn angle while clonazepam, haloperidol, and valproic acid had the opposite effect [43, 44, 46, 47]. Although speculative, the effects of MC on the parameters above may be related to the modulation of dopaminergic transmission.

Some studies have suggested that the antidepressant effects of curcumin are associated with the modulation of dopamine pathways. In mice, acute curcumin (5 and 10 mg/Kg, p.o. and 20–80 mg/kg, i.p.) produced a significant inhibition of the immobility in the tail suspension test, forced swimming, and locomotor test. At the same time, it increased dopamine, serotonin, and noradrenaline levels, inhibiting both monoamine oxidase types in the mouse brain [48, 49]. In our study, MC affected locomotion, suggesting that the micronization process increases the psychostimulant actions of curcumin in zebrafish larvae.

In the light-dark, zebrafish larvae show white preference (black avoidance). In this test, diazepam increases the time spent in the black side [30]. Our results indicate that curcumin does not present anxiolytic effects in zebrafish larvae since there was no change in time spent in the light side in LDT [30] neither in time spent in the periphery area (thigmotaxis) in the OTT [15]. Moreover, curcumin does not change the percentage of animals in the non-stimulus area in the AST. Avoidance behavior in the 7-day-old larvae is important to detect a predator in the environment and may be also an indirect measure of anxiety and cognition [15]. Indeed, curcumin does not appear to have an acute anxiolytic effect or to impair avoidance behavior. In mice, curcumin (20 mg/kg) did not demonstrate any significant anxiolytic effects in the elevated plus-maze, open field test, and forced swim test, and no interaction of curcumin at the benzodiazepine site of the $GABA_A$ receptor was observed [50]. In OTT, larvae show significantly less thigmotaxis behavior after exposure to the anxiolytic diazepam but not to the antidepressant fluoxetine [15]. In the same study, the diazepam-treated larvae showed an increased avoidance, unlike fluoxetine and caffeine-exposed larvae that displayed a decrease on this parameter. The differences between diazepam and fluoxetine are expected since these drugs target different

neuronal signaling pathways [15]. Perhaps, for these same reasons, there were no observed effects of curcumin in these behavioral tests.

Although curcumin has antioxidant effects in several studies both *in vitro* and *in vivo* [28, 51, 52], we did not find an increase in antioxidant protection related to the non-enzymatic antioxidant GSH (represented by NPSH), nor a decrease in basal lipid peroxidation levels (represented by TBARS) in zebrafish larvae. In our previous study, acute exposure to curcumin and micronized curcumin had no significant antioxidant effects in adult zebrafish. We hypothesize that these effects are only observed when there is an increase in the production of reactive oxygen species or oxidative damage, in higher concentrations of the curcumin and/or for a longer period of exposure in adult and larval zebrafish [17, 28].

CONCLUSION

In this study, MC increased locomotion and absolute turn angle but did not change anxiety, cognitive, and oxidative status outcomes in zebrafish larvae. We infer that the micronization process increases the stimulant effects of curcumin, which could be related to the indirect modulation of dopaminergic signaling. However, studies with dopaminergic antagonists and extending the exposure time and concentrations of the treatments should be performed to better characterize these results.

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Author contributions

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Conceptualization, A.S., and A.P.; Methodology, A.S., R.B., C.G.R., M.G-L., L.M.B., G.P.S.A., A.P.H. J.V.O., A.M.S., A.P.; Investigation, A.S., R.B., C.G.R., M.G-L., L.M.B., G.P.S.A.; Formal Analysis, R.B., C.G.R., A.P. and A.P.H.; Resources, J.V.O., A.M.S., A.P.; Writing - Original Draft, A.S.; Writing - Review & Editing, A.S., R.B., C.G.R., M.G-L., L.M.B., G.P.S.A., A.P.H., J.V.O., A.M.S., A.P.; Supervision, A.P.; Funding Acquisition, A.P.

Competing interests

The authors declare no competing interests.

Data availability

The datasets generated in the current study are available from the corresponding author on reasonable request.

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4. DISCUSSÃO

Os achados principais da tese relacionados aos resultados dos capítulos I, II e III são resumidos e ilustrados nas figuras 5 e 6. Em encéfalos de peixe-zebras adultos como esperado, os protocolos de estresse induziram dano oxidativo. O ECI diminuiu os níveis de GSH (NPSH), aumentou a atividade da GR e induziu peroxidação lipídica (TBARS). O EAC também diminuiu os níveis de GSH e induziu peroxidação lipídica. Nesse estudo, CUR e CM aumentaram a atividade da GPx no ECI. Além disso, CM foi capaz de bloquear as alterações no estado oxidativo induzidas por ECI, mas não por EAC. Em testes antioxidantes *in vitro*, a micronização também potencializou os efeitos antioxidantes da curcumina em parâmetros envolvendo remoção de radicais livres, podendo ser correlacionados com os efeitos protetores encontrados *in vivo*. Em parâmetros comportamentais, ECI aumentou a distância no teste de interação social e diminuiu os cruzamentos, tempo de permanência e entradas no topo no teste de tanque novo. EAC aumentou os cruzamentos, diminuiu imobilidade e aumentou tempo no centro no teste de tanque aberto. Entretanto, em nosso estudo nenhuma das preparações de curcumina bloquearam os efeitos comportamentais induzidos pelos modelos de estresse crônico e agudo.

Vários estudos demonstram que o efeito antioxidante da curcumina pode ser atribuído aos grupos fenólicos que possuem potencial antioxidante devido às suas propriedades como agentes redutores, doadores de hidrogênio, assim como inibidores de adsorção de oxigênio (ZHENG et al., 2017). Como mostrado no capítulo I, para comparar os efeitos antioxidantes *in vitro* das duas preparações de CUR e CM realizamos testes de capacidade doadora de elétrons (redutora) pelo método de FRAP, eliminação de radicais livres pelo método de DPPH, proteção contra oxidação de GSH e inibição da formação de radical hidroxila pelo método de desoxirribose. Nossos resultados indicam que a micronização potencializou esses efeitos mais evidentemente na concentração de 1 g/L, apresentando maior concentração de Fe^{2+} no FRAP, maior porcentagem de inibição do radical DPPH e da formação do radical hidroxila. Assim, demonstrando que CM possuí maior capacidade de reduzir e remover radicais livres

atuando como doadora ou na transferência de elétrons. Esses resultados serviram de base para a escolha da dose utilizada nos testes *in vivo*.

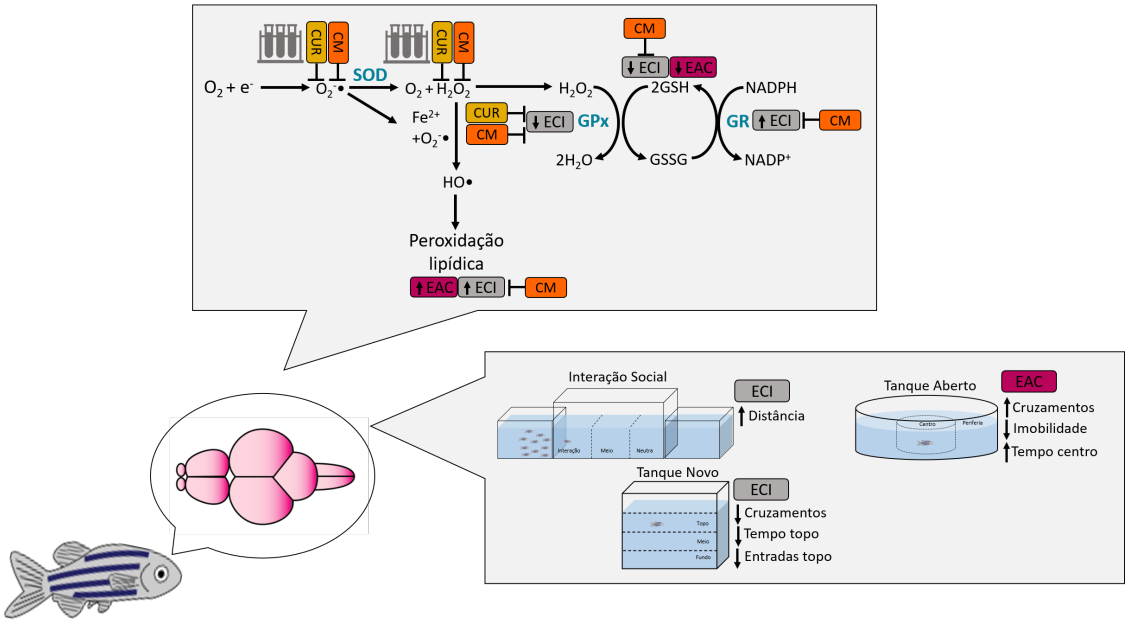

Figura 6. Resumo dos resultados obtidos na tese referentes aos capítulos I e II. ECI (estresse crônico imprevisível), EAC (estresse agudo por contenção), CM (curcumina micronizada), CUR (curcumina), GSH (glutationa), GSSG (glutationa oxidada), GR (glutationa redutase), GPx (glutationa peroxidase), SOD (superóxido dismutase), $O_2^{-\cdot}$ (ânion superóxido), $OH^{\cdot}$ (radical hidroxila), H_2O_2 (peróxido de hidrogênio), O_2 (Oxigênio), Fe^{2+} (Ferro no estado ferroso), NADPH (nicotinamida adenina dinucleótido fosfato), $NADP^+$ (nicotinamida adenina dinucleótido fosfato oxidada).

Com o objetivo de avaliar os efeitos antiestresse da CUR e CM, utilizamos modelos de estresse crônico e agudo. O modelo de ECI em peixe-zebra utilizado no capítulo I vem sendo usado em diversos estudos por apresentar validade de face (correlatos comportamentais semelhantes aos observados em humanos submetidos a situações estressantes como aumento da ansiedade) (BERTELLI et al., 2021; MARCON et al., 2016, 2019; MOCELIN et al., 2019a; PIATO et al., 2011a; REDDY et al., 2021), validade de homologia (bases neurobiológicas são semelhantes às observadas em pacientes com depressão/ansiedade como aumento de cortisol, estresse oxidativo e neuroinflamação) (CHAKRAVARTY et al., 2013; MARCON et al., 2018, 2019;

MOCELIN et al., 2019a; O'DANIEL; PETRUNICH-RUTHERFORD, 2020; PIATO et al., 2011a; REDDY et al., 2021) e validade de predição (fármacos que são utilizados na clínica para transtornos de humor e de ansiedade são efetivos nesse modelo) (DEMIN et al., 2020, 2021b; MARCON et al., 2016; REDDY et al., 2021; SONG et al., 2018). Esses efeitos foram confirmados em nosso estudo onde ECI induziu comportamentos semelhantes à ansiedade como diminuição no número de cruzamentos entre as áreas, tempo de permanência e entradas na área superior do tanque no teste de tanque novo. Além disso, ECI também induziu danos oxidativos ao diminuir os níveis de NPSH (GSH) e aumentar os níveis de TBARS (MDA). O modelo de estresse agudo por contenção (EAC) utilizado no capítulo II, também vem sendo utilizado por induzir comportamento semelhante ao da ansiedade, porém seus achados são heterogêneos. Já foi observado que EAC por 15 minutos diminui o tempo e as entradas na área superior no teste de tanque, bem como diminui parâmetros locomotores (PIATO et al., 2011b; REIS et al., 2020). Em nosso estudo, EAC por 90 minutos aumentou o número de cruzamentos, diminuiu o tempo imóvel e aumentou o tempo e permanência no centro do aparato no teste de tanque aberto. Tradicionalmente, o aumento da exploração de áreas aversivas como o centro no teste de tanque aberto é geralmente considerado um efeito ansiolítico (JOHNSON; HAMILTON, 2017; STEWART et al., 2012). Esses achados, portanto, podem sugerir que o estresse agudo pode aliviar momentaneamente a ansiedade no peixe-zebra, o que parece contraintuitivo, mas não é inédito na literatura (CHAMPAGNE et al., 2010). O mesmo foi observado em um estudo utilizando EAC por 15 minutos, apresentando aumento no número de cruzamentos e diminuição no tempo de permanência na área de tigmotaxia (periferia) no teste de tanque aberto (CHAMPAGNE et al., 2010). Em outro estudo, o EAC por 90 minutos aumentou o tempo de permanência dos animais no topo do aquário no teste de tanque novo, sugestivo de diminuição de comportamento tipo ansioso (BERTELLI et al., 2021). Supomos que o estresse agudo pode prejudicar as habilidades cognitivas agindo como um fator de desorientação, causando uma redução nos comportamentos de ansiedade e de avaliação de risco em peixes-zebra. Esses resultados contraditórios podem estar relacionados aos diferentes protocolos usados (tempo de restrição, por exemplo) nos

diferentes estudos e/ou métodos usados para quantificar o comportamento (BERTELLI et al., 2021). Além disso, EAC induziu danos oxidativos ao diminuir os níveis de NPSH (GSH) e aumentar os níveis de TBARS (MDA), corroborando com os resultados encontrados na literatura (BERTELLI et al., 2021).

Apesar dos efeitos antidepressivos da curcumina já mencionados na literatura, há poucos estudos demonstrando seus efeitos ansiolíticos em roedores, sendo somente observados em altas dosagens e em maiores tempos de exposições (BHUTANI; BISHNOI; KULKARNI, 2009; CEREMUGA et al., 2017; DA SILVA MARQUES et al., 2021; KULKARNI; BHUTANI; BISHNOI, 2008; WANG et al., 2008). Tanto em regime de exposição aguda quanto crônica, ambas as preparações de curcumina (10 mg/kg), foram incapazes de bloquear ou prevenir os efeitos comportamentais induzidos pelos diferentes protocolos de estresse, indicando que de fato a curcumina não apresenta efeitos protetores nos testes comportamentais e na dosagem utilizada em nossos estudos. Apesar disso, nosso estudo utilizando ECI, demonstrou que a curcumina chega no encéfalo por apresentar efeitos antioxidantes e esses efeitos foram potencializados pela micronização, sendo que CM foi capaz de bloquear o dano oxidativo induzido por ECI. Entretanto esses efeitos não foram capazes de bloquear os efeitos comportamentais, sinalizando que o estresse crônico pode alterar outros circuitos relacionados ao comportamento independentes de estresse oxidativo. Os efeitos antioxidantes encontrados *in vivo* correlacionam-se com os encontrados *in vitro* visto que *in vitro* CM foi capaz de reduzir íons ferro, radical DPPH e impedir a oxidação de peróxido de hidrogênio em radical hidroxila, agindo como doadora de elétrons e hidrogênio, o quais podem ser utilizados para reduzir ERO e proteger as mitocôndrias no estresse oxidativo (AK; GÜLÇIN, 2008). Entretanto, no EAC, ambas as preparações de curcumina foram incapazes de prevenir o dano oxidativo induzido por estresse agudo, o que indica que seus efeitos antioxidantes *in vivo* podem ser somente observados após exposições crônicas.

Os achados principais da tese relacionados aos resultados do capítulo III são demonstrados na figura 6. CM aumentou distância total percorrida e ângulo absoluto de virada no teste de tanque aberto, entretanto, ambas as preparações de curcumina não

apresentaram efeitos nos testes de claro/escuro e de estímulo aversivo, nem nos ensaios bioquímicos em larvas de peixe-zebra.

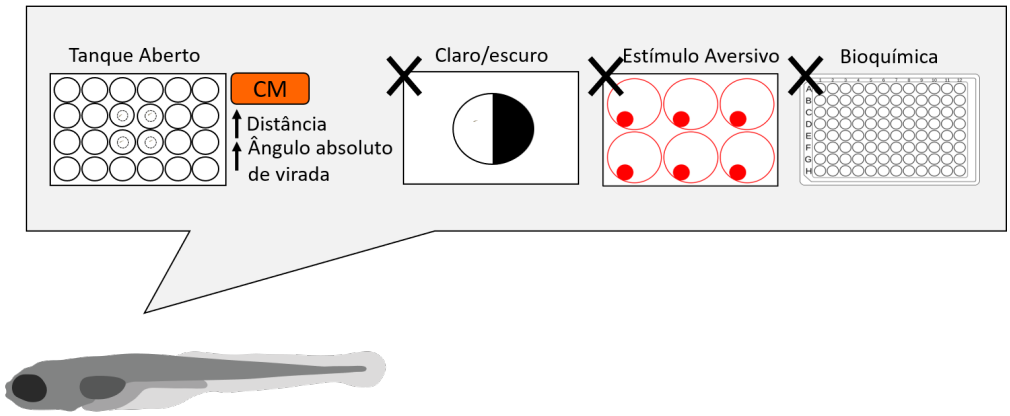

Figura 7. Resumo dos resultados obtidos na tese referente ao capítulo III. CM (curcumina micronizada).

As larvas de peixe-zebra apresentam a barreira hematoencefálica mais permeável, sendo apenas completamente estabelecida após 10 dpf, e podendo ter o comportamento e as preferências, diferentes do peixe-zebra adulto (COLWILL; CRETON, 2011; FLEMING; DIEKMANN; GOLDSMITH, 2013). Sendo assim, utilizamos larvas de peixe-zebra 7 dpf para elucidar possíveis efeitos locomotores, ansiolíticos, cognitivos e antioxidantes da exposição aguda de CUR e CM 1 µM, como demonstrado no capítulo III. Nosso estudo revelou uma alteração comportamental induzida por CM observada pelo aumento da distância percorrida e ângulo absoluto de virada no teste de tanque aberto, indicando aumento da locomoção. Esses efeitos são geralmente observados com estimulantes do SNC que afetam direta ou indiretamente a função dopaminérgica em larvas de peixe-zebra (IRONS et al., 2013). Alguns estudos indicam que a curcumina pode modular o sistema dopaminérgico (KULKARNI; BHUTANI; BISHNOI, 2008; XU et al., 2005). Isso indica um possível efeito psicoestimulante da curcumina potencializado pela micronização em larvas de peixe-zebra. Entretanto, não foram observados efeitos comportamentais tipo-ansiolíticos ou

cognitivos de ambas as preparações de curcumina nos testes utilizados. Sobre a atividade antioxidante, não foram observados efeitos *per se* de ambas as preparações de curcumina em larvas. Supomos que os efeitos antioxidantes só possam ser observados em larvas de peixes-zebra quando houver um insulto oxidativo ou em maiores concentrações. De fato, em um estudo utilizando embriões (48 horas pós-fertilização), curcumina 15 µM foi capaz de impedir os danos oxidativos induzidos por hidroperóxido de terc-butila (tBOOH), um conhecido indutor de estresse oxidativo em peixes-zebra, protegendo contra a letalidade e alterações morfológicas (ARTEAGA et al., 2021).

5. CONCLUSÃO

A micronização potencializou os efeitos antioxidantes da curcumina em testes *in vitro*, sinalizando aumento do potencial antioxidante do composto. No modelo de estresse crônico imprevisível, a micronização potencializou os efeitos antioxidantes da curcumina por bloquear os danos oxidativos induzidos pelo estresse, indicando aumento da biodisponibilidade do composto, entretanto, isso não foi suficiente para bloquear os efeitos do estresse em parâmetros comportamentais. No modelo de estresse agudo por contenção, ambas as preparações de curcumina não foram capazes de prevenir os efeitos do estresse em parâmetros comportamentais e bioquímicos, mesmo após a micronização. Em larvas de peixe-zebra 7 dpf, nossos resultados também demonstraram que a micronização potencializou os efeitos hiperlocomotores da curcumina, entretanto, não foram observados efeitos ansiolíticos, cognitivos e antioxidantes. Nosso estudo agrega importantes achados que contribuem para o corpo de evidências que apoiam a utilização do processo de micronização como estratégia para aumentar a biodisponibilidade e, potencialmente, os efeitos terapêuticos de compostos ativos.

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ANEXOS

ANEXO 1. Carta de aprovação da Comissão de Ética no Uso de Animais (CEUA-UFRGS)

U F R G S
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

PRÓ-REITORIA DE PESQUISA
Comissão De Ética No Uso De Animais

CEUA UFRGS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 35279
Título: Papel neuromodulatório da curcumina micronizada em peixes-zebra

Vigência: 07/05/2018 à 07/05/2022

Pesquisadores:

Equipe UFRGS:

ÂNGELO LUIS STAPASSOLI PIATO - coordenador desde 07/05/2018
Ricieri Naue Mocelin - Aluno de Doutorado desde 07/05/2018
Matheus Felipe Marcon - Aluno de Doutorado desde 07/05/2018
Adrieli Sachett - Aluno de Doutorado desde 07/05/2018
Ricieri Naue Mocelin - desde 01/07/2020

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 18/06/2018 - Sala 323 do Anexo I do Prédio da Reitoria, CAMPUS CENTRO/UFRGS, em seus aspectos éticos e metodológicos, para a utilização de de 2.200 adultos jovens (machos e fêmeas) e 1306 larvas de peixes-zebra (Danio renio), provenientes do biotério do departamento de Bioquímica , de acordo com os preceitos das Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008, o Decreto 6899 de 15 de julho de 2009, e as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), que disciplinam a produção, manutenção e/ou utilização de animais do filo Chordata, subfilo Vertebrata (exceto o homem) em atividade de ensino ou pesquisa. Este documento revoga a Carta de Aprovação emitida anteriormente.

Porto Alegre, Quinta-Feira, 4 de Março de 2021

ALEXANDRE TAVARES DUARTE DE OLIVEIRA
Coordenador da comissão de ética

ANEXO 2. Artigos científicos produzidos ao longo do doutorado

1. **Sachett, A.,** Benvenutti, R., Reis, C.G., Gallas-Lopes, M., Bastos, L.M., Aguiar, G.P.S., Herrmann, A.P., Oliveira, J.V., Siebel, A.M., Piato, A. Micronized curcumin causes hyperlocomotion in zebrafish larvae. ***bioRxiv*** 2021, https://doi.org/10.1101/2021.11.29.470475

2. **Sachett, A**.; Gallas-Lopes, M.; Benvenutti, R.; Marcon, M.; Aguiar, G. P. S.; Herrmann, A. P.; Oliveira, J. V.; Siebel, A. M.; Piato, A. Curcumin Micronization by Supercritical Fluid: In Vitro and in Vivo Biological Relevance. ***bioRxiv*** 2021. https://doi.org/10.1101/2021.07.08.451641.

3. **Sachett, A**.; Gallas-Lopes, M.; Benvenutti, R.; Marcon, M.; Linazzi, A. M.; Aguiar, G. P. S.; Herrmann, A. P.; Oliveira, J. V.; Siebel, A. M.; Piato, A. Non-Micronized and Micronized Curcumin Do Not Prevent the Behavioral and Neurochemical Effects Induced by Acute Stress in Zebrafish. ***bioRxiv*** 2021. https://doi.org/10.1101/2021.10.11.463974.

4. Valadas, J.; **Sachett, A**.; Marcon, M.; Bastos, L. M.; Piato, A. Ochratoxin A Induces Behavioral and Neurochemical Changes in Adult Zebrafish; ***bioRxiv*** 2021. https://doi.org/10.1101/2021.10.18.464868.

5. Benvenutti, R.; Gallas-Lopes, M.; **Sachett, A**.; Marcon, M.; Strogulski, N. R.; Reis, C. G.; Chitolina, R.; Piato, A.; Herrmann, A. P. How Do Zebrafish (Danio Rerio) Respond to MK-801 and Amphetamine? Relevance for Assessing Schizophrenia-Related Endophenotypes in Alternative Model Organisms. ***J. Neurosci. Res***. 2021. https://doi.org/10.1002/jnr.24948.

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7. Reis, C. G.; Mocelin, R.; Benvenutti, R.; Marcon, M.; **Sachett, A**.; Herrmann, A. P.; Elisabetsky, E.; Piato, A. Effects of N-Acetylcysteine Amide on Anxiety and Stress Behavior in Zebrafish. ***Naunyn. Schmiedebergs Arch. Pharmacol.*** 2020, *393* (4), 591–601. https://doi.org/10.1007/s00210-019-01762-8.

8. Bevilaqua, F.; **Sachett, A**.; Chitolina, R.; Garbinato, C.; Gasparetto, H.; Marcon, M.; Mocelin, R.; Dallegrave, E.; Conterato, G.; Piato, A.; Siebel, A. M. A Mixture of Fipronil and Fungicides Induces Alterations on Behavioral and Oxidative Stress Parameters in Zebrafish. ***Ecotoxicology*** 2020, *29* (2), 140–147. https://doi.org/10.1007/s10646-019-02146-7.

9. Garbinato, C.; Schneider, S. E.; **Sachett, A**.; Decui, L.; Conterato, G. M.; Müller, L. G.; Siebel, A. M. Exposure to Ractopamine Hydrochloride Induces Changes in Heart Rate and Behavior in Zebrafish Embryos and Larvae. ***Environ. Sci. Pollut. Res***. 2020, *27* (17), 21468–21475. https://doi.org/10.1007/s11356-020-08634-2.

10. Thiel, N. A.; **Sachett, A.;** Schneider, S. E.; Garbinato, C.; Decui, L.; Eichwald, T.; Conterato, G. M. M.; Latini, A.; Piato, A.; Siebel, A. M. Exposure to the Herbicide 2,4-Dichlorophenoxyacetic Acid Impairs Mitochondrial Function, Oxidative Status, and Behavior in Adult Zebrafish**. *Environ. Sci. Pollut. Res.*** 2020, *27* (36), 45874–45882. https://doi.org/10.1007/s11356-020-10497-6.

11. Valadas, J.; Mocelin, R.; **Sachett, A.;** Marcon, M.; Zanette, R. A.; Dallegrave, E.; Herrmann, A. P.; Piato, A. Propiconazole Induces Abnormal Behavior and Oxidative Stress in Zebrafish. ***Environ. Sci. Pollut. Res.*** 2019, *26* (27), 27808–27815. https://doi.org/10.1007/s11356-019-05977-3.

12. Mocelin, R.; Marcon, M.; D'ambros, S.; Mattos, J.; **Sachett, A**.; Siebel, A. M.; Herrmann, A. P.; Piato, A. N-Acetylcysteine Reverses Anxiety and Oxidative Damage Induced by Unpredictable Chronic Stress in Zebrafish. ***Mol. Neurobiol***. 2019, *56* (2), 1188–1195. https://doi.org/10.1007/s12035-018-1165-y.

13. Marcon, M.; Mocelin, R.; **Sachett, A**.; Siebel, A. M.; Herrmann, A. P.; Piato, A. Enriched environment prevents oxidative stress in zebrafish submitted to unpredictable chronic stress. ***PeerJ***, 2018, 6, e5136. https://doi.org/10.7717/peerj.5136.

- **Outras publicações**

1. **Sachett, A**.; Gallas-Lopes, M.; Conterato, G. M. M.; Benvenutti, R.; Herrmann, A.; Piato, A. Quantification of Nonprotein Sulfhydryl Groups (NPSH) Optimized for Zebrafish Brain Tissue. 2021. https://doi.org/10.17504/protocols.io.bx8tprwn.

2. **Sachett, A.;** Gallas-Lopes, M.; Conterato, G. M. M.; Herrmann, A.; Piato, A. Glutathione peroxidase (GPx) activity assessment for zebrafish brain tissue. 2021. https://doi.org/10.17504/protocols.io.bsujneun.

3. **Sachett, A.;** Gallas-Lopes, M.; Conterato, G. M. M.; Herrmann, A.; Piato, A. Glutathione reductase (GR) activity assessment for zebrafish brain tissue. 2021. https://doi.org/10.17504/protocols.io.bsuuneww.

4. **Sachett, A.;** Gallas-Lopes, M.; Conterato, G. M. M.; Herrmann, A. P.; Piato, A. Antioxidant Activity by DPPH Assay: In Vitro Protocol. 2021. https://doi.org/10.17504/protocols.io.btbpnimn.

5. **Sachett, A.;** Gallas-Lopes, M.; Conterato, G. M. M.; Herrmann, A. P.; Piato, A. Antioxidant Activity by Reduced Glutathione (GSH) Assay: In Vitro Protocol. 2021. https://doi.org/10.17504/protocols.io.btaynifw.

6. **Sachett, A.;** Gallas-Lopes, M.; Conterato, G. M. M.; Herrmann, A. P.; Piato, A. Antioxidant Activity by Deoxyribose Assay: In Vitro Protocol. 2021. https://doi.org/10.17504/protocols.io.btjdnki6.

7. **Sachett, A.;** Gallas-Lopes, M.; Conterato, G. M. M.; Herrmann, A.; Piato, A. Antioxidant activity by FRAP assay: In Vitro Protocol. 2021. https://doi.org/10.17504/protocols.io.btqrnmv6

8. **Sachett, A.;** Gallas-Lopes, M.; Conterato, G. M. M.; Benvenutti, R.; Herrmann, A.; Piato, A. How to Prepare Zebrafish Brain Tissue Samples for Biochemical Assays. 2020. https://doi.org/10.17504/protocols.io.bjkdkks6.

9. **Sachett, A.;** Gallas-Lopes, M.; Conterato, G. M. M.; Benvenutti, R.; Herrmann, A.; Piato, A. Protein Quantification Protocol Optimized for Zebrafish Brain Tissue (Bradford Method). 2020. https://doi.org/10.17504/protocols.io.bjnfkmbn.

10. **Sachett, A.;** Gallas-Lopes, M.; Conterato, G. M. M.; Benvenutti, R.; Herrmann, A.; Piato, A. Quantification of Thiobarbituric Acid Reactive Species (TBARS) Optimized for Zebrafish Brain Tissue. 2020. https://doi.org/10.17504/protocols.io.bjp8kmrw.